A study of the effect of activated peripheral blood mononuclear cell derived cytokines upon monocyte mediated tumour cytotoxicity

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The work on which this thesis is based is my own independent work except where acknowledged

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ABSTRACT

Cytokine activated peripheral blood mononuclear (PBMC) cells, sometimes called 'Lymphokine Activated Killer (LAK)' cells, have been shown to mediate tumour cell cytotoxicity in vitro but their mode of action in vivo is poorly understood. LAK cell therapy in humans on the whole has been unsuccessful with only minor success against melanomas and renal cell carcinomas. However the mode of tumour regression in these few instances is still unknown. In vitro tumour cytotoxicity by LAK cells does occur but LAK cells do not traffic to, accumulate around or infiltrate the site of tumours in vivo. Intravenous infusion of LAK cells into peripheral circulation causes the cells to traffic to the liver and spleen for clearance. The spleen is a lymphoid cell reservoir and thus interaction with T cells and monocytes would be common. Therefore these cellular effector cells may be causing tumour cell regression via an indirect route, meaning they are activated by secondary cytokines produced by the LAK cells. This project investigates the hypothesis that cytokines known to be produced by LAK cells can induce an anti-tumour response upon monocytes in vitro.

The aim of this project was firstly to investigate the effect of cytokines secreted from activated (PBMC) upon monocyte mediated tumour cell cytotoxicity. More specifically it was to determine the exposure time and concentration of mitogen, which would cause LAK cells to optimally secrete secondary cytokines which in turn would induce monocyte tumour cell cytotoxicity. Analysis of cytokine secretion allowed the dose and exposure time of stimulus (either IL-2 or anti-CD3 antibody) which generated the cytokine rich supernatants to be defined. Three cytokines were examined, in the supernatants, to determine whether they had any effect on monocyte tumour cytotoxicity, using a 48hr ^{3}H-Uridine cytotoxicity assay. Their concentrations were determined using bioassays and ELISA's. The cellular mechanism of monocyte tumour cytotoxicity was examined by observing adhesion molecule upregulation (in particular LFA-1 and Mac-1), MHC II cell surface density and also TNF-α secretion by the monocytes. This was carried out to create a model of how these secondary cytokines were actually destroying the tumour cells.

In conclusion, recombinant IL-2 and soluble monoclonal anti-CD3 both induced LAK activity and cytokine secretion in normal PBMC. Furthermore, secondary cytokines, particularly IL-1 and IFN-γ, produced by the IL-2 stimulated cells induced both an increased TNF-α secretion and tumour cytotoxicity by monocytes. These two parameters were found to be directly proportional to one another. These results imply that secondary cytokine production by IL-2 activated PBMC causes increased tumour cell killing by monocytes in vitro. The cytokines IL-1 and IFN-γ were found to be important in the upregulation of monocyte cytotoxicity and the cytokine TNF-α was found to be important in the direct mechanism of tumour cell killing. The adhesion molecules examined did not appear to have a role in the tumour cell killing. However other cytokines, mitogens and/ or adhesion molecules may also be involved and further study would be required to determine if this were the case.

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ABBREVIATIONS

Antigen Presenting Cell	APC
Antibody Dependent Cellullar Cytotoxicity	ADCC
Bovine Serum Albumin	BSA
Cluster Determinant	CD
Counts per minute	CPM
Chromium-51 sodium chromate	^{51}Cr
Enzyme Linked Immunosorbent Assay	ELISA
Ethylene diamino-tetra acetic acid	EDTA
Fraction antibody binding	Fab
Constant (Crystallizable) Fragment	Fc
Foetal Calf Serum	FCS
Fluroscien Isothiocyanate	FITC
Interferon	IFN
Interleukin	IL
Intracellular Adhesion Molecule	ICAM
Leukocyte funtion Antigen	LFA
Lipopolysaccharide	LPS
Lymphokine Activated Killer	LAK
Major Histocompatability Complex	MHC
Mega Bequerel	MBq
Monoclonal antibody	mAb
Monocyte tumour cytotoxicity	MTC
Natural Killer	NK
Peripheral blood mononuclear cell	PBMC
Phosphate Buffered Saline	PBS
Tritiated-Uridine	^{3}H
Tumour Necrosis Factor	TNF

CHAPTER 1

INTRODUCTION

1.1 Cancer and the immune system

1.2 Cytokines

1.2.1 Interleukin-1

1.2.2 Tumour Necrosis Factor -α

1.2.3 Interferon-γ

1.3 Lymphokine Activated Killer Cells (LAK) cells

1.4 Monocytes / Macrophages

1.5 Adhesion Molecules

1.6 Destruction of tumour cells by monokines

1.1 : Cancer and the Immune System

Scientists through the ages have attempted to understand how the body coordinates its fight against disease, in the hope that understanding such processes would lead to new treatments. Much is now known about the cells in that fight. The immune system of man contains a wide diversity of cell types with differing functions and morphology. The major immune cells are the monocytes/ macrophages, the lymphocytes and the neutrophils (1).

Monocytes are phagocytic cells which travel around the body in the circulation and upon stimulation (usually with particulate materials) are able to pass from blood vessels into surrounding tissue, thereby changing morphology and becoming macrophages. Antigen is first taken up by such phagocytic cells, processed in a complex fashion and re-expressed on the surface of the cell in association with class II Major Histocompatability Complex (MHC) molecules. Antigen can then be recognised by the T lymphocytes, notably CD4+ cells.

The lymphocytes can be separated into two basic sub-populations: cells derived from the thymus are denoted " T cells". One of the main functions of these cells, together with those cells presenting antigen, is to recognise newly processed antigen and MHC and produce growth factors, differentiation factors and signals for antibody secretion which act upon the other main populations of lymphocytes , those denoted as "B cells". "B cells" ere derived from stem cells in the bone marrow and their main function is to produce antibody against the primary antigen (2).

These growth and differentiation factors produced by T cells and other immunocytes were found to be proteins and named cytokines (cytokines also have a variety of other names; being lymphokines, monokines, interleukins etc.). Their structure, function and physiology have been ever since a major research topic, as they play a central role in the fine regulation of the immune system. Cytokines are proteins which act as chemical messengers in the body. They are produced by a wide range of cells and have varied effects. The response elicited by a particular cytokine may vary from cell to cells. Cytokines are able to deliver a number of different message to cells, or they can enhance a single message depending upon the particular cytokine, which cell it acts on and what other messages the cell is receiving at that time (3).

Each type of cytokine has its own particular receptor and the presence or absence of a receptor determines whether or not the corresponding cytokine can affect the cells. Once there has been binding between the cytokine and its receptor a signal is relayed to the nucleus of

the cell, causing the cell to induce or repress a particular response via protein expression , thus changing the cells behaviour. Only very small concentrations of cytokines are required to produce a large response. they were therefore initially very hard to examine but now, with the help of recombinant DNA technology and genetically engineered bacteria , pure cytokines can be manufactured in large quantities. Scientists have identified several types of cytokines e.g. the interleukins, tumour necrosis factor (TNF), interferons and the haemopoietic growth factors (colony stimulating factors, CSF) etc., but other cytokines (as yet undiscovered) may play as vital a part in immune regulation (4).

The first real positive result using cytokines as an anti- tumour therapy were observed by Mule and Rosenburg (5). IL-2 was used to activate splenocytes , producing lymphokine activated killer (LAK) cells, which were able to lyse a broad spectrum of fresh tumours in short term chromium release assays. Injections of LAK cells and recombinant IL-2 were able to mediate regression of established pulmonary metastasis from a variety of sarcomas , a carcinoma and a melanoma. However other types of murine tumour required a much higher concentration of IL-2 to cause regression and high doses of IL-2 were found to have a detrimental effect on the body. Therefore the cure , and its side effects (capillary leak syndrome and its cascade effects), were as harmful to the body as the disease.

Lymphocytes were then removed from cancer patients and stimulated with IL-2, producing human LAK cells, which were subsequently reintroduced to the patient together with IL-2 (6,7). This too cause some tumour regression without so much of an increased cytotoxic effect but the therapy was not working on all or even most of the patients, thus the question is raised why is this so?

To summarise this:

1) IL-2 and LAK are synergistic in mice

2) Even in mice, some tumours (less immunogenic) need higher doses of IL-2 than others.

3) The mouse tumour model is unrealistic because of:

a)immunogenic tumours

b) very early age at the start of therapy

c) IL-2 intolerance

4) Humans are unable to tolerate equivalent IL-2 doses to mice (wt/wt) and therapy was less successful.

5) Human evidence is that the vast proportion of LAK cells do not traffic to tumours: their mode of action may therefore be secondary to their effects on the host immune system.

In vitro it was known that the LAK cells were capable of mediating a cytotoxic effect but was their a secondary cytotoxic tumour effect due to LAK cell cytokine secretion upon other cells of the immune system? If secondary cytokine are required in tumour cell killing what is their optimal non harmful concentration and is a synergy between cytokines needed?

The aim of this project was to investigate the ability of LAK products to stimulate cells of the immune system to kill tumour cells. The cytotoxic cells observed for stimulation by activated PBMC supernatants in this study , were peripheral blood monocytes because they had been shown by Ruco to be tumour cytotoxic under certain stimulation.

1.2 Cytokines

LAK cells produce a wide variety of cytokines. Three were chosen for this study which were in our opinion, on examining the current literature, the most likely to effect monocytic stimulatory capabilities. These cytokines were IL-1, TNF-α, and IFN-γ:

1.2.1 : Interleukin-1

Interleukin 1 (IL-1) is a highly pleiotropic cytokine released primarily from activated monocytes and macrophages. It was first identified in 1972 when it was observed that phytohaemagglutinin (PHA) or lipopolysaccharide (LPS) stimulated the release of some factors by adherent cells in culture and that supernatants from such cultured cells, in combination with PHA, could stimulate thymocyte proliferation (8). Initially named "Lymphocyte Activating Factor" (LAF), IL-1 is called by many other names which describe the proteins different reported activities. Two forms have been isolated : IL-1α and IL-1 . Both polypeptides , of approximate M_r17kD, occur naturally in human, pig, rabbit and mouse exhibit a high degree of inter-species homology (for example 62% homology between human and mouse IL-1 a (9)).

IL-1a and IL-1 B represent the products of two distinct genes and is initially synthesised as 30-32kD propeptides of 270 and 269 amino acids respectively. Mature forms of IL-1a and IL-1 b are represented by C-terminal cleavage products of 159 and 153 amino acids respectively. Both IL-1 precursors lack the hydrophobic signal peptide thought to be required for extracellular transport of proteins manufactured within the cell (10). Biochemical data suggest the existence of a membrane bound form of IL-1 (11). However, immunochemical data indicated that IL-1 is only found associated with the cytoplasmic ground substance of the activated monocyte (12). The mechanism of IL-1 secretion is unknown. IL-1α and IL-1β exert their effects through a specific 80-82kD receptor (12). The receptor has been found to have near identical affinity to each form of IL-1, therefore both IL-1 proteins and their IL-1 precursors all compete equally for receptor binding (13).

A number of cell types have been shown to produce IL-1 , including monocyte and macrophage cell lines, Langerhans cells, natural killer cells (NK), B cell lines, endothelial cells etc. (9).

Some reported IL-1 activities include:

1) Induction of chemotaxis in polymorphonuclear cells (PMN)

and macrophages.

2) Induction of endothelial cell proliferation.
3) Pro-coagulant activity.
4) Enhancement of Type IV collagen production by epidermal cells.
5) Induction of osteoblast proliferation
6) Stimulation of bone resorption by osteoclasts.
7) Modulation of reparative functions following tissue injury.

Perhaps the most important effects of IL-1 are those related to immune function. Some examples include (14):

1) Induction of cytokine release by T cells.
2) Co-stimulation of B cell differentiation and proliferation.
3) Augmentation of NK mediated cytotoxicity.
4) Induction of chemotaxis, degranulation and release of neutrophils from the bone marrow.
5) Induction of prostaglandin release, chemotaxis and tumour cell lysis by macrophages.
6) Secretion of acute phase proteins by hepatocytes.
7) Induction of fever.

It has also been shown that IL-1 stimulates the release of factors associated with the growth and differentiation of cell associated with myeloid and lymphoid cell lineage's in vitro . For example, IL-1 may induce the production of Granulocyte colony stimulating factor (G-CSF) and macrophage colony stimulating factor (M-CSF) by human bone marrow stromal cells (15).

T cell activation with IL-1 has produced some controversial results. In 1985 IL-1 was observed to produce an accessory signal in the activation of T cells. Furthermore evidence from studies using tumour cell lines suggests that IL-1 may induce or up regulate factors required for T cell activation and proliferation (e.g. IL-2, IL-2 receptor). However, other evidence suggests IL-1 is not required, but rather serves to enhance the stimulatory function of accessory cell in T cell activation (16).

The activities attributed to IL-1 suggest several potential therapeutic applications including use as a vaccine adjuvant, as a wound healing agent or as a stimulator for haematopoesis in immunodeficiency. However, the role examined in this study is its effect upon peripheral blood monocytes in conjunction with tumour cell killing and whether activated PBMC may contribute to this.

1.2.2 : Tumour Necrosis Factor-Alpha (TNF-α)

TNF-α was discovered following the observation that the serum from mice injected with Bacillus Calmette Guerin (BCG) or LPS induced tumour necrosis in tumour bearing mice. TNF-α has also been termed Cachectin, because of its role in inducing a wasted or cachetic state through its inhibition of the enzyme lipoprotein lipase (21).

TNF-α is a 17kD polypeptide existing in trimer units and containing several possible glycosylation sites. Cloning data suggest human TNF-α is a 157 amino acid polypeptide, processed from non-reducing electrophoresis studies shows a single 17kD band , suggesting that covalent interactions are not involved in multimer formation (3).

As well as the secreted form, a membrane -bound form of TNF-α exists which appears as a 26kD polypeptide translation product and includes a 9kD leader sequence. Although this leader sequence is removed co-translationally to yield the 17kD secreted form it apparently persists in membrane TNF (22). The primary source of TNFα is the activated macrophage whereas TNF-β (lymphotoxin-α) it is the activated B cell. TNF-α and TNF-β share identical actions including:

1) Osteoclast activation and bone resorption
2) Antiviral activity.

However in contrast to TNF-α, TNF-β is unable to induce the release of haematopoietic growth factor activity from endothelial cells in vitro. TNF-α has been shown to be the principal mediator of toxic shock syndrome . TNF-α apparently shares several bio-activities previously ascribed IL-1 (23) or colony stimulating factors from a variety of sources. TNF-α Also appears to work synergistically with other cytokines (24).

1.2.3 Interferon-γ α(IFN-γ)

The interferons (IFNs) were originally identified and characterised according to their ability to induce the production of RNA and protein in target cells. Type I or viral interferons including: IFN-α (leukocyte derived) and IFN-β (Fibroblats derived), and are produced during viral or bacteria infection. Type II or immune interferon (IFN-γ) is produced primarily by Natural Killer cells upon stimulation with mitogens or antigens.

In this study we examined the effect of IFN-γ on the stimulation o monocytes to enhance tumour cell killing. In humans IFN-γ is produced by the CD4+ and CD8+ T lymphocytes, in response to antigen stimulation; this can be modelled in vitro with either recombinant interleukin-2 (r.IL-2) or anti-

CD3 antibodies. IFN-γ increases the number of high affinity receptors for IgG (FcRI) on monocytes and macrophages. This has important effects in terms of removal of immune complexes, phagocytosis and antibody-dependent cellular cytotoxicity (ADCC). IFN-γ has also been shown to activate oxidative metabolism in monocytes and macrophages and to be essential for clearance of intracellular organisms, such as Listeria, which grow in macrophages.

Human IFN-γ is a polypeptide with a molecular weight of 35-70kD according to molecular sieve data and with a pI of 8.6-8.7. Cloning data suggest that IFN-γ mRNA code for a 166 amino acid polypeptide of 17.1kD containing a 20 residue N terminal hydrophobic signal peptide and two potential glycosylation sites. Glycosylation at Asn 38 is associated with a lower molecular weight form (20 kD) whereas glycosylation at both Asn 38 and Asn 100 yields a heavier form (25kD) according to SDS -PAGE analysis . It appears that in humans glycosylation has some effect on competitive binding of IFN-γ to its receptors (27). The 50-70 kD species initially reported may represent a multimeric IFN-γ (26).]

IFN-γ exerts its biological effects through specific, saturable binding to a single class of high affinity receptors. IFN-γ receptors can be found in a number of different types of tissues including myelomonocytic cells, lymphoid cells, mast cells , endothelial cells, fibroblasts, neuronal cells, and melanocytes. Receptors for IFN-γ may vary between tissues i.e. those receptors present on monocytes are found to be different to those present in non haemopoietic tissue. Other than its antiviral activity IFN-γ has various other activities which include (28):

1) Inhibition of cell growth in the presence of lymphotoxin (TNF-beta)
2) Induction of MHC class I and II expression in target tissues.
3) Priming macrophages for tumour cell killing and enhancing NK cytotoxicity (29,30)
4) A possible role in the induction of cytolytic activity in LAK and CTL cells by up regulating the p55 component of the IL-2 receptor.
5) Up regulation of the secretory component which is required for both binding and transport of secreted IgA, IgM and IgG Fc receptors on PMN
6) An important role in B cell differentiation and has been

shown to induce or enhance Ig secretion in resting B lymphocytes

IFN-γ has a number of possible clinical uses in the treatment of psoriatic arthritis and in the early stages of hepatitis infection. The most publicised uses for IFN-γ have been those involving cancer treatment but the results of such experiments on the whole have been disappointing. For example, IFN-γ has been tested in phase I and II clinical trials in combination with antibodies to metastatic colorectal carcinoma cells. The treatment is based on ADCC effected by the combination of IFN-γ and the antibody CD17-1A, and has been shown to be efficacious in vitro. The poor response seen in patients may reflect the inadequate distribution of antibody sites of interest in vivo.

Therefore IFN-γ is only waiting for pharmacological advances, towards reducing systemic toxicity and enhancing targeting methods in vivo, to offer a great deal to the treatment of certain clinical disorders.

1.3 : Lymphokine Activated Killer (LAK) Cells

Tumour cells may be lysed by the host immune system in a number of ways. These include :

1) Natural Killer Cytotoxicity (NK). This type of killing is MHC-unrestricted, does not involve memory and therefore represents non-acquired innate immunity. Lysis is restricted to a select group of transformed cell lines: fresh solid tumour cells and tumour cell lines are mostly unaffected.

2) Activated Cytotoxic Lymphocytes (CTL). This involves recognition of a specific antigen (via MHC) and consequently represents memory-related immunity. On paper this may seem an excellent way to deal with the tumour but until the recent discovery of the MAGE antigen family which are present on melanomas and are recognised by autologous CTL was close to impossible.

3) LAK Mediated Cytotoxicity. This is another type of cytotoxicity which is brought about by the LAK cell (68). The LAK cell is produced by activating existing lymphocytes, or thymocvtes, using IL-2 or other known mitogens. The progenitors of LAK activity in human peripheral blood are mainly Large Granular Lymphocytes (LGL) with the phenotype CD3-, CD16+, NKH-1. However, it is now known that such variables as growth medium, stimulatory mitogen (and its concentration), phenotypic composition of starting cultures and duration of culture may influence the capacity of lymphocyte subsets to generate LAK activity and the phenotype of the effectors generated (69). Experiments have been carried out to show that this killing is distinct from NK killing with the use of NK resistant (Daudi) and sensitive target cells (K562).

Some typical properties and functions of LAK cells are (32):

1) Fresh solid tumour cells are resistant to lysis by autologous PBL containing NK cells, but are lysed by autologous LAK cells;

2) Fresh solid tumour cells are resistant to lysis by fresh NK containing PBL of normal individuals;

3) Fresh tumour cells are lysed by allogeneic LAK cells from cancer patients and normal individuals;

4) Autologous T Cell Growth factor (TCGF/IL-2) stimulates LAK lytic action for autologous tumour;
5) Interferon has been shown to augment NK activity and to stimulate lysis of allogeneic but not of autologous fresh tumour;
6) LAK effectors are non-adherent and express serologically detectable T cell markers i.e. CD3+ but also NK cell markers e.g. CD56 and CD57;

The LAK phenomenon appears to be of particular interest because of the potent ability of IL-2 to both stimulate cytotoxic activity and to promote the expansion of the effector cell population. The early work was carried out in mice and it was found that these experiments required large doses of IL-2. The equivalent doses in humans were never reached due to toxicity but even reduced doses were found to be toxic, causing fever, chills, malaise, fluid retention and mild hepatic dysfunction. While fever, chills and malaise can be controlled with drugs such as acetaminophen and indomethacin, the problem of fluid retention is more difficult to treat (33,34). Instead of injecting large (and sometimes lethal) doses of cytokine into patients it was found that the cells which the IL-2 was stimulating in vivo could be removed from the body, stimulated in vitro and then replaced after activation (7).

LAK cells can be generated easily from peripheral blood lymphocytes, the lymphoid cells being obtained by leukapheresis or thoracic duct drainage. These cells can then be incubated with IL-2 and then reintroduced back into the body. A large amount of IL-2 is required per treatment/ experiment, for example a typical in vitro stimulatory dose for a mouse is 175,000 Units of r.IL-2/ $5x10^6$ splenocytes(7) and further in vivo doses of IL-2 must be given in addition to this once the cells are reintroduced to the body. This is because the cells depend on the continued presence of IL-2 for their survival e.g. Donohue and Rosenberg. demonstrated that the serum half life of IL-2 was only 3 minutes (35).

Another type of activated lymphocyte can be produced by stimulation using the monoclonal antibody (mAb) anti-CD3 which binds to the T cell receptor (TCR) complex (70). The stimulated cell then has the ability to become cytotoxic to tumour cells and secrete various cytokines (71). This method of producing activated lymphocytes is useful as LAK cell therapy but it requires a lot of PBMC. Therefore in children in particular, due to their smaller blood volume, this is not a practical method. Anti-CD3 therapy combined with IL-2, results in a large increase in cell yield and the development of high LAK activity (72). LAK cell therapy has only been proven to work in a small proportion of individuals, showing a great intra species variation, in this response. Unfortunately in humans, only melanoma

and renal cell sarcoma have demonstrated any real regression with LAK cell therapy (33). It has been shown that LAK cells do not accumulate at or infiltrate into metastatic lesions (78) and that following infusion into peripheral circulation they traffic through the lungs to the liver and spleen for clearance (34). The spleen is a lymphoid cell reservoir and thus interaction between LAK cells, T cells and monocytes would be common. Therefore the LAK cells may be causing tumour cell regression indirectly, via local secretion of secondary cytokines with other cells of the immune system.

1.4 : Monocytes/Macrophages

Monocytes and macrophages are two of the main cellular phagocytes of the immune system, together with the neutrophil polymorphs. The blood monocyte is a long lived cell which is able to migrate into the tissues and differentiate into the tissue macrophage. Metabolically, macrophages are more active than monocytes, they are strongly phagocytic (due to increased numbers of surface receptors) and produce more types of lysosomal enzymes). Most monocytes and macrophages express the CD14 molecule on their cell membrane; however , there, can be great phenotypic ranges of monocytes within the body (37).

All nucleated cells express the cell surface proteins MHC I (i.e. HLA A,B,C etc.) and therefore can act as targets for class I restricted T cells. The primary role of MHC I restricted T cells is the recognition and destruction of virally infected, transformed or otherwise abnormal cells. Monocytes and macrophages also present antigen in association with MHC class II molecules. Macrophages are phagocytic and possess a number of degradative enzymes. They are particularly effective at processing and presenting antigen , such as bacteria and certain parasites and play a key role in triggering T cell mediated immunity towards intracellular parasites and facultative anaerobic bacteria. They accomplish this by presenting antigen of the infected host cells, which they have recently engulfed and digested, on their cell membranes in conjunction with MHC II T hese antigens derived from foreign bodies would thus trigger T cell recognition etc. Receptor mediated internalisation of immune complexes , via Fc and complement receptors can markedly enhance macrophage antigen presentation.

Over eighty five years ago Elie Metchikoff noted that mononuclear phagocytes from animals which were resistant to certain bacterial infections, had an increased ability to engulf and destroy the afore-mentioned microbes. In the 1970's Evans & Alexander and Hibbs & Remington found that these activated macrophages (whose morphology is different from that of the normal macrophage) were also capable of mediating anti-tumour effects (37).

Onozaki (38) stated that human peripheral blood monocytes from normal donors showed considerable cytotoxicity against tumour cells when pre-incubated in vitro for 24 hr's with purified, human monocyte derived IL-1. It has also been reported that IL-1, which is produced primarily by monocytes (39), appears to be chemotactic for human monocytes and may

therefore function as an auto-stimulating signal. In addition IFN-γ can also increase the secretion of TNF-α (40, 41) and monocyte mediated cytotoxicity of tumour cells. (42). In fact, IL-1, IFN-γ and TNF-α have all been demonstrated to be inducers of TNF-α mediated tumour cytotoxicity by monocytes (43,44). Furthermore it appears that monocytes become primed upon stimulation by certain cytokines and require a further stimulus (i.e. cell to cell contact) to bring about lysis (42,45).

Thus a complex pattern of events first reported by Ruco in 1978 (45) is required. The events appear to be as follows. Blood derived monocytes are activated with cytokines. Cytokine responsiveness, however, is labile and gradually lost therefore further signals are required for expression of cytotoxic activity. expression signals (such as adhesion molecules), from the tumour cells, provide the final stimulus for macrophage cytotoxicity against tumour cells. Another example of this complex pathway is; resting macrophages express low levels of MHC class II but these levels are markedly increased by cytokines such as IFN-γ (46). At a site of possible inflammation or infection T-cells could secrete IFN-γ which could activate macrophages to secrete a variety of protease's into their local environment. This process could to some degree, degrade antigenic material sufficiently (without further processing) for presentation by other class II bearing cells. Thus in our experiments not only did we examine the effect the cytokines had on the tumouricidal properties of the monocytes but also the density of MHC class II (HLA-DR) present on the monocytes both pre and post cytokine stimulation.

1.5 : Adhesion Molecules

Adhesive interactions between cells play a central role in the function of the immune system. Cell to cell contact between monocytes and tumour cells may enhance cytotoxicity through specific interactions between surface proteins an the two cell types. In the resting immune system these proteins must be at a base level otherwise cells expressing this same target receptor would be altered or destroyed by the monocyte. However, under certain stimuli the receptors on the monocyte (or other effector cell) may be increased and produce a heightened effector-target cell interaction.

Bernasconi et al (47) discovered a correlation between the leukocyte beta-2 integrin and tumouricidal activity of the human monocyte. The integrin in question is made up of two proteins, CD18 and CD11 (48). Later Jonjic (52) reported that the adhesion and cytotoxicity of human monocytes activated by IFN-γ and LPS against epithelial cells involves CD18. Thus, this integrin appeared to be important in monocyte tumour cytotoxicity and worth study. There are a number of forms of this integrin, the two we chose to examine in our study were lymphocyte functional related antigen-1 (LFA-1) and Mac-1. LFA-1 is expressed on monocytes and granulocytes, as well as t and B lymphocytes (48,49). It is composed of two proteins CD11a and CD18 and is thought to be induced by IFN-γ (50). The LFA-1 receptor on the target cell is ICAM-1 or ICAM-2 (intracellular adhesion molecule) (51), both are members of the immunoglobulin supergene family.

Mac-1 is also a leukocyte beta-2 integrin as it is composed of the same CD18 protein but in contrast the second protein is CD11b. It is expressed on monocytes, neutrophils and NK cells. This protein is also known as the CR3 (Complement-3-receptor). It binds proteins containing the sequence Arg-Gly-Asp notably the C3 and C4 components of complement (in a Ca^{2+} dependent manner). This causes the release of oxidative intermediates i.e. H_2o_2 and superoxide. Expression of the protein on the cell membrane of monocytes depends on the activation state of the cell (47).

1.6 : Destruction of Tumour Cell by Monokines

Human peripheral blood monocytes may become tumouricidal when incubated with endotoxins or cytokines. After stimulation, macrophages are known to produce over 100 distinct molecules from the superoxide anion (32D) to the protein fibronectin (444kD) which are able to bring about diverse effects from cell division to tumour lysis (53). Several cytokines are secreted by activated macrophages, of which IL-1 and TNF-α have both been shown to produce tumour cell damage, singly (Lachman et al 1986, (54)) and in synergy (55). IFN-γ has been reported to produce oxygen intermediates e.g. superoxide which are also cytotoxic to tumour cells.

Project aim

This project is aimed to examine the use of LAK cells not as cytotoxic effector cells, but as cytokine secreting cells which might have the ability to stimulate secondary effector cells of the host immune system and so thereby inducing fewer of the toxic effects seen with the in vivo use of IL-2. LAK cells may secrete different cytokine profiles depending on amount of IL-2 pre-incubated with them and the duration of pre-incubation. The correct time and dose must therefore be determined to produce endogenous cytokines which have the desired cytotoxic effect.Then once knowing the cytokine profile of particular supernatants which enhanced monocyte tumour cytotoxicity, we could then examine in what ways this cytotoxicity was achieved. To this aim TNF-α levels were measured in activated monocytes to examine the correlation between their possible heightened concentration and tumour cell killing.

CHAPTER 2

INTRODUCTION

1.1 General Laboratory Equipment

2.2 Tissue Culture Medium and Target Cell lines

2.3 Radioactivity and separation medium

2.4 Cytokines and Mitogens

2.5 Antibodies

2.6 Hardware

2.7 List of Suppliers Names and addresses

2.1 : General Laboratory Equipment

1) Chemicals and Glassware, Unless otherwise stated all general laboratory chemicals and glassware were of the highest purity and quality available.

2) Heparin. The anticoagulant heparin was obtained from the hospital pharmacy. It was diluted to 100 IU/ml in PBS and stored at 4°C. Heparin was added to fresh blood, for PBMC separation, to prevent the blood from clotting.

3) Plastic disposables. Universals were purchased from Sterilin Ltd. 24-well and 96-well flat-bottomed tissue culture grade plates were purchased from Greiner. Syringes and needles were purchased from Becton Dickinson. All tissue culture flasks were purchased from Greiner and BDH.

4) Trypan Blue powder, was bought from BDH chemicals Ltd. A 1% (w/v) solution was prepared in PBS and solid residue was removed by filtration. The solution was autoclaved at 10lb/ sq in. for 10mins.

5) Phosphate Buffered Saline (PBS) tablets, were bought from Sigma Chemicals Ltd. and added to the prescribed volume of water; the resulting solution was then autoclaved.

6) Bovine Serum Albumin (BSA) was bought from Sigma and stored at 4°C.

2.2 : Tissue Culture Medium And Target Cell Lines

1) L-Glutamine, 200mM was obtained from Gibco, and stored in 1ml aliquots at -20 C.

2) RPMI-1640 medium was purchased from Gibco in 500ml bottles and stored at 4'C. Before use, 1ml of 200mM L-glutamine was added to l00ml of medium.

3) AIM V medium, bought from Gibco and stored in 100ml aliquots at 4°C in the dark.

4) K562 Cell Line was obtained from the Surgery Dept. the Western Infirmary Glasgow and was maintained as an adherent cell line in RPMI-1640 medium plus 10% foetal calf serum. It is a leukaemic cell line (Lozzio and Lozzio 1975).

5) SW742 Target Cell Line, a colon adenocarcinoma cell line (Leibovitz et al 1976) was a generous gift from Dr. Russell Greig of Smith, Kline and Beecham and maintained as an adherent cell line in RPMI-1640 medium plus 10% foetal calf serum.

6) U937 Effector cell line, was obtained from the Surgery Dept. Western Infirmary Glasgow. It is a non-adherent diffuse histocytic lymphoma line, which expresses many monocyte-like characteristics exhibited by cells of histocytic origin and was maintained in DMEM (Gibco) plus 10% foetal calf serum.

7) Dl0(N4)M cell line obtained from Dr SJ Hopkins, Rheumatic Disease Centre, Hope Hospital, Salford M6 8HD, UK

8) Trypsin/EDTA was purchased from Gibco, diluted to 0.25% (v/v) in PBS and stored at 4 C.

9) EDTA was purchased from BDH at 2.5%(v/v) and made to 0.02% (w/v) in PBS, autoclaved at 10lb/sq in. for 10min and stored at 4°C.

2.3 : Radioactivity and Separation Medium

1) ^{51}Chromium was obtained from Amersham International Ltd as a stock solution of sodium chromate with an activity of 185MBq in 1ml. This was diluted to a volume of 5ml i.e. 37MBq/ml. l00ul of this solution was then used to label the target cells for a LAK cytotoxicity assay.

2) ^{3}H-Uridine was purchased from Amersham International Ltd. with an activity of 185MBq in 1ml. This was diluted to a volume of 5ml i.e. 37MBq/ml.

l00ul of this solution was then used to label the target cells for a monocyte cytotoxicity assay.

3) Scintillation Fluid was obtained from Packard

4) Lymphocyte Separation Medium, Nycoprep was obtained from Nycomed Ltd. It was stored in the dark at room temperature. Its density is 1.077g/ml.

5) Monocyte Separation Medium, Nycodenz Monocytes was obtained from Nycomed Ltd. It was stored in the dark at room temperature. Its density is 1.068g/ml.

2.4 : Cytokines and Mitogens

1) Recombinant human Interleukin 2 was a gift from Mr. P. McCulloch Dept. Surgery, Western Infirmary, Glasgow.

2) Lipopolysaccharide was bought from Sigma Chemicals Ltd. in quantities of 1mg . This was resuspended in PBS and stored, at 4°C as 200ng/ml aliquots.

2.5 : Antibodies

1) Anti CD3 Antibody (SHL45.6) was a bivalent monoclonal which was used to produce the LAK cells, it was obtained from Dr M Clark, Dept. Pathology, Cambridge University.

2) Anti CD14 (UCHM1 IgG) was obtained from the Scottish Antibody Producing Unit in 1ml of culture supernatant, produced in a mouse. The antibodies Immunoglobulin Class/ Isotype was IgG_{2A}. It was stored at 4°C and used neat.

3) Anti CD3 was obtained from the Scottish Antibody Producing Unit in 1ml of culture supernatant, produced in a mouse. The antibodies Immunoglobulin Class/ Isotype was IgG_1. It was stored at 4°C and used neat.

4)Anti CD19 was obtained from the Scottish Antibody Producing Unit in 1ml of culture supernatant, produced in a mouse. The antibodies Immunoglobulin Class/ Isotype was IgG_1. It was stored at 4°C and used neat.

5)FITC anti -mouse IgG was raised in sheep. The IgG fraction of sheep anti-mouse IgG is purified by treatment of the antiserum with n-octanoic acid followed by ion exchange chromatography. The immunoglobulin is conjugated to FITC and the conjugate is separated from unreacted FITC by gel filtration. It was obtained from the Scottish Antibody Producing Unit with a protein content of 9.8mg/ml. It was stored at 4°C and used neat.

6) NKH-1 was obtained from Dako in a 2ml aliquot of culture supernatant produced in a mouse. Its Isotype was IgG1. It was stored at 4°C and used neat.

7) LFA-1 , antiCD11a was produced from clone B-B15 , a mouse hybridoma of spleen cells from BALB/c mice and the mouse myeloma cell line X63/AG.8653, and obtained from Serotec in a volume of 2ml of purified Ascites.The antibodies isotype is IgG_1. It was stored at 4°C and used as recommended at 10ul per test.

8) Mac-1 , antiCD11b was produced from clone 44 , a mouse hybridoma and obtained from Serotec in a volume of 2ml of purified Ascites.The antibodies isotype is IgG_1. It was stored at 4°C and used as recommended at 10ul per test. (approximate Isotype concentration is 50ug/ml , determind by radial immunodiffuusion.

9) Anti MHCII DA6.231 was obtained from Keith Guy, Dept Immunology, University of Strathclyde. It was stored at 4°C and used for flow cytometry at the recommended concentration of a 1:10 dilution of stock.

10) Anti-TNF-α antiserum, $1x10^4$ neutralising units /ml of ascites was a gift from Dr. R Rees and used in his laboratory at concentrations quoted.

11) Anti-Interferon (gamma) antiserum, $1x10^5$ neutralising units /ml of ascites was a gift from Dr. R Rees and used in his laboratory at concentrations quoted.

12) Anti-Interleukin 1 antiserum, $1x10^6$neutralising units /ml of ascites was a gift from Dr. R Rees and used in his Laboratory at concentrations quoted.

2.6 : Hardware

1) Flow cytometer was available within the Surgery Dept. from Coulter Electronics Ltd.

2.7 : List of suppliers names and addresses

Amersham International Ltd.
UK Sales Office, Lincoln Place, Green End, Aylesbury,
Bucks. HP20 2TP

Becton Dickinson Ltd.
Between Towns Road, Cowley, Oxford. OX4 3LY

Biological Industries Ltd.
56 Telford Road, Cumbernauld, Glasgow G67 2AX

Boehringer Ingelheim
Boehringer Ingelheim, Bender and Co., Vienna, Austria

British Biotechnology Ltd.
Brook House, Waltington Road, Cowley, Oxford OX4 5LY

DAKO Ltd.
16 Manor Courtyard, Hughenden Ave, High Wycombe, Bucks HP31 5RE

ECACC
PHLS Centre for applied Microbiology and Research, Porton Down,
Salisbury, SP4 OJG

Gibco Ltd.
P.O. Box, Washington Rd., Abbotsinch Industrial Estate,
Paisley, PA3 4EP

Imperial Cancer Research Fund Laboratories
P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX

Nycomed Ltd.
Nycomed House, Lampton Rd., Hounslow, Middlesex, TW3 4EE

Mr P. McCulloch
Dept of Surgery, Western Infirmary, Byres Road, Glasgow, Scotland

Miltenyi Biotec
1250 Oakmead Park, Suite 210, Sunnyvale, CA 94088-3599 USA

Dr. R.C. Rees
Dept. Clinical and Experimental Microbiology, The Medical School,
Royal Hallamshire Hospital, Sheffield.

Scottish Antibody Production Unit (SAPU)
Law Hospital, Carluke, Lanarkshire, Scotland. ML8 5ES

Serotec
22 Bankside, Station Approach, Kidlington,
Oxford. OX5 1JE

Sigma Chemical Co. Ltd.
Fancy Road, Poole, Dorset BH17 7NH

Smith, Kline & Beecham
Sweedlaand, PA, USA

Sterilin Ltd.

Lampton House, Lampton Rd., Hounslow, Middlesex TW3 4EE

CHAPTER 3

METHODS

3.1 **Separation of Peripheral Blood Lymphocytes (PBL's) from Whole Blood.**

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3.3 **Detection and Analysis.**

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3.6 **Cytokine Quantification Assays.**

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3.6.2 Interferon-gamma ELISA.

3.6.3 Interleukin-1 Bioassay.

3.1 : separation of Peripheral Blood Lymphocytes (PBLs) from Whole Blood

Fresh blood (10ml, 60ml or 120ml) was obtained from normal volunteer donors. For every 10ml of blood 0.lml of Heparin was added. An equal amount of PBS was then added to the fresh blood. The resulting suspension was then layered onto density gradient medium in a ratio of 2:1 (blood:lymphoprep). It was important not to allow the two layers to mix so the lymphoprep was added first to the 20ml centrifuge tube (universal tube). The tube was then tipped almost horizontal and the blood was layered on to it using a pipette.

The universals containing blood and lymphoprep gradient were then centrifuged at 400g for 30 minutes (this step separates the lymphocytes from other blood constituents). The centrifugation was carried out at room temperature and the brake on the centrifuge was switched off to allow a good separation. From here all the reagents and samples were kept on ice. The PBL layer was removed using a pasteur pipette.

To enhance the extraction process it was found that removal of the serum layer produced a greater yield of PBLs. The PBLs removed were placed into a new (pre-cooled) universal. This universal was then filled to 1cm from the brim with cold PBS.

The universal was then placed into a cooled centrifuge (4°C) and spun at 1900 rpm for 15 mins, with the brake on (this spin was to remove any excess lymphoprep). After this centrifugation the supernatant was carefully poured off and the pellet was resuspended in a small amount of PBS. Once the pellet was resuspended, the universal was topped up with PBS.

The universal was then centrifuged at 1550 rpm for 6 mins with the brake on. The supernatant was then poured off again and the pellet was resuspended as above and the universal was topped up with PBS. The universal was recentrifuged at 1550 rpm for 6 mins (brake on). The supernatant was then poured off and the pellet was then resuspended in 1-4ml of growth medium (AIM-V) (56).

3.2 : ^{51}Cr release Assay

Experimental Theory

PBMC which are incubated with IL-2 or anti CD3 become activated, becoming LAK cells, and these activated cells can cause tumour cell lysis. Differing amounts of stimulation and periods of incubation time can bring about different levels of activation.

When a tumour target cell, which contains ^{51}Cr, is lysed by a LAK cell, it releases ^{51}Cr into the supernatant. This is then analysed using the gamma counter; i.e., only ^{5l}Cr from lysed cells is analysed, giving a value in counts per minute (CPM). The wells containing target cells alone, with the detergent Triton-X-100 (maximum release wells), gives us a result of the total incorporation of ^{51}Cr by the target cells. The target cells in RPMI alone give the lowest CPM recorded, as any CPM registered is only due to spontaneous lysis by the target cells themselves e.g. no controlled cell killing (32,60). A formula to determine the %cytotoxicity generated is given below.

The formula used to calculate the % generated cytotoxicity is:

$$\%\text{Cytotoxicity Generated} = \frac{\text{CPM Experimental-CPM spontaneous x100}}{\text{CPM Max Release -CPM spontaneous}}$$

Methodology

Target Cells

The target cells (TC) were centrifuged at 400g for 10min, then resuspended in 1ml of growth medium (RPMI 1640). The cells were counted, using a haemocytometer, and adjusted to the correct density i.e. $1x10^6$ cells/ml. 2MBq of ^{51}Cr was then added per $1x10^6$ cells and incubated with the cells at 37°C for lhr, then washed by centrifuging three times in RPMI 1640 at 400g for 10min. Finally, the cells were resuspended in 1ml of RPMI 1640 plus 10% FCS, counted and adjusted according to LAK cell number i.e. E:T ratio. The effector to target ratio used was 25 effectors to 1 target.

Effector Cells (LAK)

After removing the LAK cells from the flask 2ml of cold RPMI was added to the flask to remove any adherent cells. The cells were then washed three times in RPMI, centrifuging each time for 10mins at 400g. The cells were then resuspended in 1ml of medium, counted and then diluted to give the correct E:T ratio (see above).

Addition of Effectors to Targets

0.1ml of the effector cells (LAK) were added to three wells of a flat-bottomed 96-well plate (per test); the results obtained are a mean of this triplicate. 0.1ml of target cells were then added to each experimental well. At the bottom of the plate targets were placed into two sets of six wells. To one of these sets was added 0.1ml of medium i.e. RPMI 1640; this set of wells was called the Spontaneous release wells. To the other six wells 0.1ml of 10%(v/v) solution of Triton-X-100 (Sigma), a detergent, was added, these wells were known as the Maximum release wells. A mean result was also calculated for the Spontaneous release and the Maximum release. The plate was incubated for four hours at 37°C, 5%$C0_2$.

After this time period 0.1ml of the supernatant was carefully removed from each well and placed into a separate gamma counter tube. The tubes were placed into a gamma counter and the number of counts per minute (CPM) emitted by each tube was recorded over a two minute period. Note that when the 0.1ml of supernatant was removed from the well, only liquid was removed i.e. all the cellular material from the assay remained on the bottom of the well.

3.3 : Detection and Analysis

Flow cytometry was used to determine the purity of cell types and/or contaminating cell populations and observe up or down regulation of certain cell surface proteins.

To detect particular protein receptors present on the cell membrane a very sensitive instrument was required. The instrument used in our experiments was called an EPICS, manufactured by Coulter. This machine, together with mouse anti-human primary antibodies (which bind to specific human antigens) and goat anti-mouse secondary antibodies (which have the fluo rochrome FITC conjugated to their Fc region and bind to the mouse antibodies), made up the flow cytometric requirements to examine the proteins present on PBMC and monocytes.

The EPICS is a four component system consisting of :

1) A light source, which is a laser.
2) A sample changer and optical assembly.
3) A set of associated electronics that convert light impulses into digital signals.
4) A printer to dump relevant information from the screen onto paper.

Cells to be analysed were labelled with fluorochromes and placed into the flow cytometer at a density of $5x10^5$ cells/ml. The light from the laser is scattered by the cell. While the light is scattered in 360°, the amount of light scattered in the forward direction (along the axis of the light beam) is approximately proportional to cell size. The magnitude of this parameter, known as forward angle light scatter (FALS), is directly proportional to the size of the particle if the particle is a homogeneous sphere.

Lymphocytes (which are small cells with relatively homogenous nuclei and a high nucleus to cytoplasmic ratio) have low FALS, whereas granulocytes and monocytes, with larger more complex nuclei tend to have greater FALS. Also, the large angle light scatter (LALS) can be measured. This value coincides with the amount of light reflected by the internal structures of the cell, therefore granulocytes etc. have higher LALS and lymphocytes have lower LALS.

Fluorescence is the second event, occurring when a fluorochrome-bearing cell absorbs the laser light at incident wavelength and then re-emits the light at a longer wavelength. The emitted light is at a lower energy and is thus a different colour. As with scatter, the fluorescent light is emitted through

360° but collected through an optical detector located orthogonally to the light beam. If several different fluorochromes are present in or on the cell, light of several different wavelengths is emitted at the same time and detected with a series of optical filters. This multicoloured emission may be detected by a different detector. The emission from different fluorochromes can then be separately quantified using a photo multiplier and a computer.

The results are divided into two categories:

i) The "mean channel" denotes the average proportion of fluorescence present per cell i.e. receptor density per cell. It must be noted that these figures are averages with 50% of the population having higher and 50% having lower receptor densities than this value.

ii) The "% cells" stained denotes the proportion of cells stained with at least one fluorochrome (appear fluorescent) with relation to the whole population of cells.

On average 10,000, cells were passed through the EPICS before a reading was taken. This was carried out so that the results were representative.

3.3.1 : FLOW CYTOMETRY STAINING PROTOCOL

Cells (monocytes or PBMC) obtained from whole blood using the protocols already described were analysed for their purity and/or the presence of certain protein receptors present on their surfaces by flow cytometry.

For each flow cytometric analysis $5x10^5$ cells were required. The cells were placed into a lml "Eppendorf" tube and 500ul of PBS (containing 1ml of BSA/100ml PBS) was added. The BSA prevented FITC from sticking non-specifically to the lymphocytes. The tube was centrifuged for two minutes at 600g and 4°C (this was the first wash). The supernatant was poured off carefully and the pellet resuspended. The 1° monoclonal antibody was then added (see Table 1 for specificities and volumes used) and the tube was incubated at 4°C (in an ice bucket) for 15-30 minutes.

PBS was then added as above (second washing). The tube was then centrifuged at 600g and 4°C. The supernatant was then poured off carefully and discarded. The pellet was resuspended and the 2° monoclonal antibody i.e. conjugated fluorescent marker (FITC) was then added. The tube was

then incubated at 4°C for 15-30 minutes. Again PBS was added as before and the tube was recentrifuged and the supernatant was discarded. The pellet was then resuspended in approximately 0.2ml of PBS (1% BSA v/v) solution and then the sample was placed into the EPICS (65).

Note: Gloves were worn at all times.

All reagents were kept cold (4°C)i.e. cells, BSA and PBS.

When a control was used only FITC (2° monoclonal)and not 1 monoclonal was added to the cells.

Table 1

Amount of neat 1° antibody added per 5x105 cells

Monoclonal	Volume ul /5x105 cells	Specificity
Anti CD3	100	Binds to 'T' cells
Anti CD14	100	Binds to monocyte
Anti CDI9	100	Binds to B cell
NKH-1	5	Binds to NK cell CD56
HLAI	10	Binds to MHC class I
HLAII	5	Binds to MHC class II
LFA-1	10	Binds to monocyte CDIIa
Mac-1	10	Binds to Monocyte CD11b

Note: All the antibodies were all used neat.

3.4 : Separation Of Peripheral Blood Monocytes

Experimental Theory

Monocytes were to be the effector cells in this system so a methodology was required to separate these cells from whole blood. In general, monocyte cell numbers are only a tenth of the PBMC population and thus the normal $1x10^6$ PBMC/ml of blood translates into $1x10^5$ monocytes/ml of blood. This yield is quite small and therefore large quantities of blood would be required to perform the cytotoxicity assays. Thus the best separation possible was required. Three techniques were examined A) Separation media (59) B) Adherence (57) C) MACS (58)

Experimental Methodology

3.4.1 :Density Separation on "Nycodenz Monocytes"

Blood was collected into syringes where one volume 2.7%(w/v) EDTA (pH 7.4) has been added prior to collecting nine volumes of whole blood. Ten parts EDTA/blood were m1xed with one part 6%Dextran 500 in 0.9% (w/v)NaCl. The plasma layer was removed when the erythrocytes had settled (15-30min at 37°C). The leukocyte-rich plasma was then layered over Nycoprep 1.068 in 2:1 ratio. The tubes were centrifuged for 15min; 600g at room temperature. After centrifugation, the clear plasma was removed down to 3-4mm above the interface, and discarded. Thereafter the remaining plasma, together with slightly more than half the volume of separation fluid was collected and washed in 0.9% NaCl containing 0.13% EDTA and 1% heat inactivated Foetal Calf Serum (FCS) to remove contaminating platelets. The monocytes were washed four times in this solution the first centrifugation being 600g and subsequent centrifugation's at 200g. The monocytes were then counted and the resuspended to a cell density of $1x10^6$ cells/ml.

3.4.2 : Adherence

$50x10^6$ PBMC (separated using Lymphoprep) were resuspended in 20ml of RPMI 1640 plus 10% FCS. These cells were incubated for 90min in a 175cm tissue culture flask at 37°C. Non adherent cells were gently removed and the plates were washed five times in warmed PBS. After microscopic examination to confirm the removal of non adherent cells, adherent cells were recovered by incubation in 5mM EDTA for 30 min at 37°C.

3.5 : Monocyte Cytotoxicity Assay

Experimental Theory

Freshly-isolated monocytes were incubated with the various LAK supernatants to bring about their activation and hopefully increase their ability to cause tumour cell lysis. To quantify this a monocyte cytotoxicity assay must be performed. The procedure for this is similar to the ^{51}Cr release assay but here ^{3}H-Uridine is used as the radioactive label because of its reduced toxicity. This must be taken into consideration bearing in mind that monocyte tumour cytotoxicity is a lot slower than LAK cell cytotoxicity, with incubation periods of 48hrs and 4hrs respectively. ^{51}Cr can show toxic effects on both the target and effector cell populations if incubated with them for as little as ten hours.

When a tumour target cell, which contains ^{3}H, is lysed by a monocyte, it releases ^{3}H into the supernatant. After an incubation time of two days the wells containing the activated monocytes and the radioactivity labelled targets cells were washed to remove any radioactivity present from lysed tumour target cells. The living (and therefore adherent) monocytes and tumour target cells were then lysed using a detergent, with the lysate from each well being collected separately and analysed using a beta counter to determine how much radioactivity remains in each well i.e., only ^{3}H from intact cells is analysed, this gives a value in counts per minute (CPM). The target cells +Triton-X-100 (maximum release wells) gives us a result of total ^{3}H uptake by the target cells, as all the targets are lysed by the detergent releasing their ^{3}H into the supernatant. Control monocytes, which were unstimulated, were also added to the labelled targets. This produced a CPM result proportional to unstimulated killing, which had to be taken into consideration as different donors monocytes were in differing states of activation (prior to LAK supernatant activation) (42,45,60). The formula to calculate the % Generated cytotoxicity is given below:

$$\%\ \text{Generated cytotoxicity} = \frac{\text{Control release - Experimental release X100}}{\text{Control release}}$$

This assay was also used using the U937 Cell line instead of monocytes to show that the effect of the supernatants was not just an artefact of a specific donor's monocytes.

Experimental Methodology

DAY1

Effectors

Monocytes were separated from whole blood using Nycoprep 1.068 (see method page). The cells were resuspended in RPMI 1640 and 10% FCS and counted using a haemocytometer. The cells were then adjusted to a density of $1x10^6$ monocytes/ml in RPMI medium. 100ul of this cell suspension was added to each well of a flat-bottomed, 96-well plate. To the wells containing the monocytes either 50ul of supernatant plus 50ul of medium or 100ul of medium (for control) was added. The cells were incubated overnight at 37°C, 5% CO2.

Targets

The (SW742) target cells were grown until 20-50% confluent. 1uCi of tritiated (^3H) uridine was then added per ml (lx106 cells) of medium and incubated overnight at 37°C,5% $C0_2$.

DAY 2

Effectors

The effector plate containing the monocytes was turned upside down on a piece of tissue paper. RPMI 1640 was then used to wash all the wells of the plate twice, turning out the plate onto tissue paper each time. The target cells were then added to the plate.

Targets

The flask containing the target cells was washed twice with a little RPMI 1640 to remove excess isotope and dead cells. 5mls of 0.02% EDTA was added to the flask containing the adherent target cells and the flask was then incubated for 5mins. After this time the flask was gently tapped 3-4 times and then the cells which were now non-adherent were removed and centrifuged with a little RPMI and 10% FCS at 1540 rpm for 6mins. 1ml of

RPMI 1640 and 10% FCS was added to the cells which were then stained with Trypan blue and counted using a haemocytometer. The target cells concentration was then adjusted to 1 x 10^5 TC/ml. 200ul of this cell suspension was added to the previously washed plate containing the monocytes. The plate was then incubated for 48hrs at 37°C, 5% $C0_2$.

Controls

target cells= 100% CPM (maximum release)

monocytes+ target cells= unstimulated killing

supernatant+ monocytes+ target cells= experimental result

Note:LPS was also used on occasion to stimulate the monocytes prior to stimulation with the LAK supernatants to enhance supernatant activation, it was used at 20ng/ml. Supernatants in certain cases were also m1xed, prior to addition to the monocytes, with anti-cytokine antisera to remove that specific cytokine from the supernatant

DAY 5

All of the wells of the plate were washed twice with RPMI 1640 turning the plate upside down each time. (If the monocyte supernatants were required for further analysis i.e. TNF ELISA, they were removed prior to plates being washed). 200ul of 10% TRITON-X-100 was then added to each well of the plate. The plates were placed in a 37°C incubator for 30mins. After this incubation the wells were aspirated 2-3 times with a pipette and 100ul of the contents of each well was placed into a separate scintillation vial. The vials were then placed into a Beta scintillation counter and each vial was read for 3mins.

3.6 : Cytokine Quantification Methodology

The amount of TNF-alpha and Interferon-gamma present in the individual supernatants was assessed using an in-house ELISA in the Dept of Clinical and Experimental Microbiology, Sheffield University with the permission and supervision of Dr C McIntyre.

The IL-1 bioassay was carried out in the Department of Medicine, the Royal Infirmary, Glasgow with the permission and supervision of Dr P Winstanley. All reagents used in the assays were provided by the afore mentioned laboratories.

3.6.1 : TNF-alpha ELISA

Each well of a 96 well immunoassay plate (GIBCO) was coated with 50ul of goat anti TNF polyclonal antibody diluted 1/500 in 0.05M carbonate buffer pH 9.6. The plate was incubated for 2hrs at 37°C in a humidified box. 150ul of TBS-BSA (2.5%) was then added to each well and the plate was incubated at 4 C overnight (i.e. BLOCKING STEP). The next day the plate was washed three times with TBS-Tween(0.1%). Standard TNF was then taken and diluted to the required concentrations of 100,75,50,25,10,5 and 1 units/ml. 50ul of standard or sample was then added to each well in duplicate and the plate was the incubated for lhr at 37°C. After this incubation the plate was washed three times in TBS-Tween (0.1%). 50ul of anti-TNF monoclonal antibody (101/4), diluted l/1000 using TBS-FCS (1%), was added to each well and the plate was again incubated for lhr at 37°C. The plate was then washed three times in TBS-Tween(0.1%). 50ul of biotinylated anti-mouse Ig (RPN1001), diluted 1/1000 in TBS-FCS (1%), was then added to each well. The plate was then incubated for lhr at 37°C. After washing the plate again three times using TBS-Tween (0.1%) 50ul of Streptavidin-alkaline phosphatase (RPN1234) was added per well (RPN1234 was diluted l/500 in TBS-FCS (1%)). The plate was incubated for a further 30mins at 37°C and then washed three times using TBS-Tween. 50ul of alkaline buffer was then added per well followed by 50ul of phosphatase substrate (4mg/ml in water). The plate was then incubated at 37°C, in the dark, until sufficient colour had developed (approx. 20min). The plate was then read using a plate-reader reading at 414nm (61).

3.6.2 : Interferon-gamma ELISA

Each well of a 96 well immunoassay plate (GIBCO) was coated with 50ul of goat anti IFN-γ polyclonal antibody diluted 1/500 in 0.05M carbonate buffer pH 9.6. The plate was incubated for 2hrs at 37°C in a humidified box. 150ul of TBS-BSA (2.5%) was then added to each well and the plate was incubated at 4 C overnight (i.e. BLOCKING STEP). The next day the plate was washed three times with TBS-Tween(0.1%). Standard IFN-γ was then taken and diluted to the required concentrations of 100,75,50,25,10,5 and 1 units/ml. 50ul of standard or sample was then added to each well in duplicate and the plate was the incubated for lhr at 37°C. After this incubation the plate was washed three times in TBS-Tween (0.1%). 50ul of anti-IFN-γ monoclonal antibody (101/4), diluted l/l000 using TBS-FCS (1%), was added to each well and the plate was again incubated for lhr at 37°C. The plate was then washed three times in TBS-Tween(0.1%). 50ul of biotinylated anti-mouse Ig (RPN1001), diluted 1/1000 in TBS-FCS (1%), was then added to each well. The plate was then incubated for lhr at 37°C. After washing the plate again three times using TBS-Tween (0.1%) 50ul of Streptavidin-alkaline phosphatase (RPN1234) was added per well (RPN1234 was diluted l/500 in TBS-FCS (1%)). The plate was incubated for a further 30mins at 37°C and then washed three times using TBS-Tween. 50ul of alkaline buffer was then added per well followed by 50ul of phosphatase substrate (4mg/ml in water). The plate was then incubated at 37°C, in the dark, until sufficient colour had developed (approx. 20min). The plate was then read using a plate-reader reading at 414nm (62,63).

3.6.3 : Bioassays for IL-1

Experimental Theory

The bioassay used was to measure the concentration of IL-1 in the LAK supernatants was carried out in the laboratory of Dr P. Winstanley, The Dept of Medicine, The Royal Infirmary, Glasgow. The assay is based on the proliferative response of the D10(N4)M cell line (Source: Dr SJ Hopkins, Rheumatic Disease Centre, Hope Hospital, Salford M6 8HD, UK) which has absolute dependency on IL-1 as a growth factor. The dose-response curve of the D10(N4)M cells to IL-1 is made much steeper by the incorporation of IL-2 into the assay. The addition of a saturating IL-2 dose increases the specificity of the assay for IL-1 by neutralising any effect of IL-2 in the test sample. The amount of proliferation was determined using an MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. When MTT is added to viable cells they reduce the soluble tetrazolium salt to give a purple precipitate. This precipitate can then be solubilised and quantified using a spectrophotometer.

Experimental Methodology

Eight serial (1 in 3) dilution's were made of each LAK supernatant, in a flat-welled micrometer plate, making a final volume of 50ul/well. The diluent used was basal medium (see below). Each supernatant was assayed in duplicate . For the controls a serial three fold dilution was made of the r.IL-2 (60U/ml) and a supernatant obtained from PBMC which had been mitogen (silica) stimulated were used as controls (thus containing various cytokines). D10(N4)M cells were harvested and washed once in basal medium. The cells were resuspended at a density of $1x10^5$ cells/ml. The medium for the IL-l assay was supplemented with 60 units/ml of r.IL-2 (Glaxo 1500U/ml). 50ul of this cell suspension was then added to each well and incubated at 37°C for three days.

10ul of MTT solution was then added to each well of the flat-bottomed microtitre plate containing 100ul of culture medium. The plate was incubated for 2hrs at 37°C .100ul of DMSO (Sigma) was then added to each well. The plates were then m1xed gently for 2-3 min on a microtitre plate shaker. The purple precipitate slowly dissolved, therefore the plates in general were left overnight at room temperature. Next day when the reduced formazan was fully dissolved the plates were read at 540nm(64,65).

D10 medium : RPMI1640 +20mM HEPES
+ 10%FCS + 1% Pen. Strep.
+ 2mM Glutamine
+ 7.5% Na_2C0_3
+ 3ug/ml Concanavalin A (con A)
+ 50uM 2-Mercaptoethanol

MTT : 3-[4,5-Dimethylthiazol-2-yl]-2,
5-diphenyltetrazolium bromide;
Sigma M2128.
dissolved at 5mg/ml in saline, then
filtered through a 0.22um filter.

CHAPTER 4

RESULTS

4.1 **PBMC activation to produce LAK cells and Cytotoxicity assay using ^{51}Cr.**

4.2 **Analysis of the LAK supernatants to determine their cytokine profiles.**

4.3 **Which Monocyte separation technique to use?**

4.3.1 Adherence Method.

4.3.2 Nycodenz Monocytes.

4.4 **What effect do the LAK cell supernatants have on monocyte tumour cytotoxicity as determined by the monocyte cytotoxicity assay.**

4.5 **What role do adhesion molecules play in monocyte mediated tumour cytotoxicity?**

4.6 **How do the activated monocytes destroy tumour cells?**

4.1 : PBMC activation to produce LAK cells and Cytotoxicity assay using ^{51}Cr

120ml of fresh venous blood was donated by each of four young healthy volunteers. Nycoprep was then used to obtain peripheral blood mononuclear cells from the whole blood (Section 3.1). The PBMC from each individual donor were then separated equally into twelve culture flasks. Four of these flasks were labelled day 1, four labelled day 4 and four labelled day 8. To the four flask for each day the appropriate amount of IL-2 was added i.e. 0,200,1000 or 2000 Units/ml of $1x10^6$ cells. The flasks were then incubated for the appropriate number of days at 37°C, 5%CO_2. After the desired incubation time, the contents of the flasks were centrifuged with the supernatants being removed and stored, in 3ml aliquots at 37°C. The cell pellet was resuspended and the cells counted and adjusted to a concentration of $25x10^5$ cells/ml. These cells were then used as effector cells in a cytotoxicity assay against target cell SW742 at a concentration of lx105 /ml i.e. E:T ratio was 25:1. 0.1ml of effectors were added to a 96 well plate and to this was added 0.1ml of target cells which had been pre-incubated with ^{51}Cr (2MBq of Cr per $1x10^6$ target cells). The cell were incubated for 4hrs (SeeSection 3.2 for remainder of cytotoxicity assay protocol).

This whole experiment was repeated with the same four donors two weeks later but instead of using IL-2 to stimulate the cells the antibody anti-CD3 was used at concentrations of 0,1,10 and l00ng/ml (in solution). The supernatants were stored as before and the cells were analysed for their killing ability using a ^{5l}Cr release assay (See above) using both SW742 and K562 cell lines as target cells.

This assay was essentially carried out to make sure that the cells were still alive after there incubation with mitogen and thus the supernatants came from viable healthy cells.

The cytotoxicity results obtained showed this to be the case but can only be looked at in a qualitative way as the target cells did not take up the radioactive chromium very well and therefore the maximum release was quite low. Nevertheless killing was seen. (Results not shown).

Because ninety six different supernatants were produced a easily recognisable code had to be derived. This code is used in the presentation of all of the following data. A key to the code is shown below.

EXAMPLE

W . X . Y . Z

W = Donor A,B,C or D
X = IL-2 (I) or Anti CD3 (C) stimulated
Y = Number of days the PBMC were stimulated for (1,4,8 days)
Z = Concentration of mitogen added per ml of media i.e.
For IL-2 --- 0,200,1000 or 2000 U/ml media
For anti CD3 --- 0,1,10 or 100 ng/ml of media

Therefore

AI10 = Supernatant derived from Donor A PBMC stimulated with zero Units/ml of IL-2 for one day.

And

DC8100 = Supernatant derived from Donor D PBMC stimulated with one hundred ng/ml of anti-CD3 for eight days.

4.2 : Analysis of the LAK supernatants to determine their cytokine profiles

The amount of three cytokines, thought to be important in the activation of monocytes, had to be determined in each of the LAK supernatants. IL-I was determined by bioassay (detailed on page50). TNF-alpha and Interferon-gamma concentrations were assessed using ELISA (see Sections 3.6.1 and 3.6.2). Of these two methods the bioassay measures only active protein whereas the ELISA measures inactive as well but the bioassay is less quantitative than the ELISA. Ideally it would have been technically correct to carry out both kinds of assay, per cytokine, on each supernatant but time unfortunately ruled out this option.

Eventually a panel of "cytotoxic promoting" supernatants was ascertained from all of the initially produced supernatants. These "cytotoxic promoting" supernatants were used primarily in subsequent experiments to reduce the vastness of each assay.

Figures 1a, 1b,1c and 1d show the data obtained from the relevant ELISA's and Bioassays.

Figure 1a depicts the cytokine profile data of the IL-2 produced supernatants from donors A and B. As can be seen from the graphs, donor B supernatants contain quite a low amount of IL-1 but even so it and donor A produce more IL-1 per incubation time at the 200 U/ml dose. Also predominantly the 200 Unit and also the 1000 Unit doses of IL-2 produced the greatest amount of Interferon gamma.

Figure 1b depicts the data for IL-2 produced supernatants from donors C and D. For the IL-I concentrations present in DonorC LAK supernatants there appears to be no real trend but, as seen in donors A and B, donor D shows the highest IL-I concentrations when stimulated with 200 U/ml and the 200 unit and 1000 unit doses also produce the highest Interferon gamma concentrations. When looking at the TNF concentrations of the four donors it can be seen that donor C LAK cells secreted a lot more TNF-alpha than those of the other three donors with the unstimulated LAK cells of each day having the highest concentrations. Donor D LAK supernatants contain quite low levels of TNF-alpha.

Figure 1c depicts the data for anti CD3 produced supernatants from donors A and B. The IL-I concentrations present in donor A LAK supernatants were greatest per day in those supernatants produced from

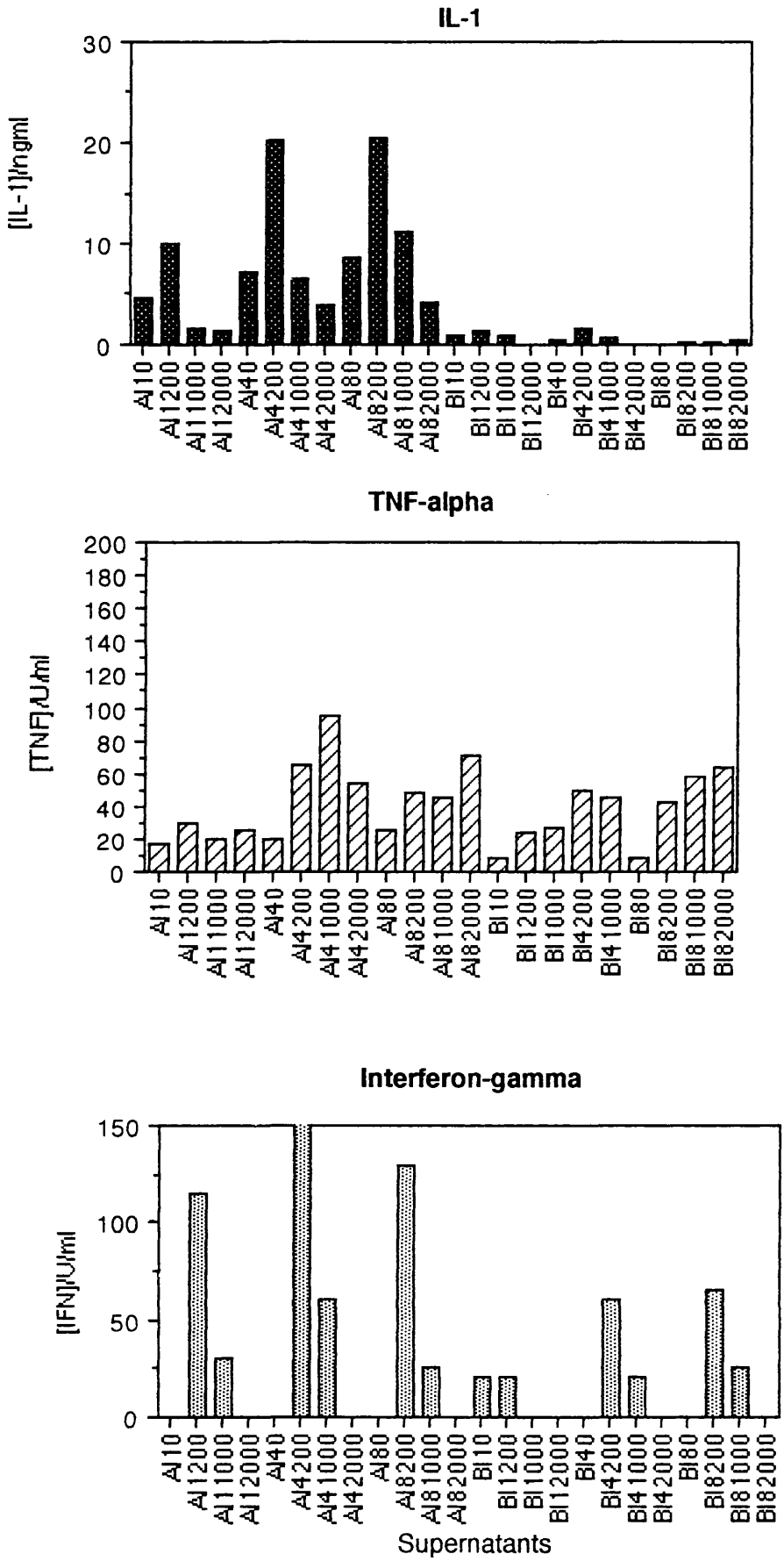
PBMC incubated with the 10ng and 100ng per ml doses of anti-CD3 and the 10ng/ml dose for donor B produced the highest concentrations for each day of incubation. Looking at the presence of TNF-alpha and Interferon-gamma for donors A and B it can be seen that per day, predominantly, the 10ng dose produces the greatest response.

Figure 1d depicts the data for anti-CD3 produced supernatants from donors C and D. Again as for donors A and B the IL-1 concentration is invariably highest when the PBMC have been incubated with the 10ng/ml dose. The concentration of TNF-alpha in donor C supernatants is again high (compare donor C, anti CD3 and IL-2, induced TNF-alpha production) with no real trend being observed. There is also no trend in TNF-alpha secretion for donor D. for Interferon-gamma presence in donor C and D supernatants there is no trend observable.

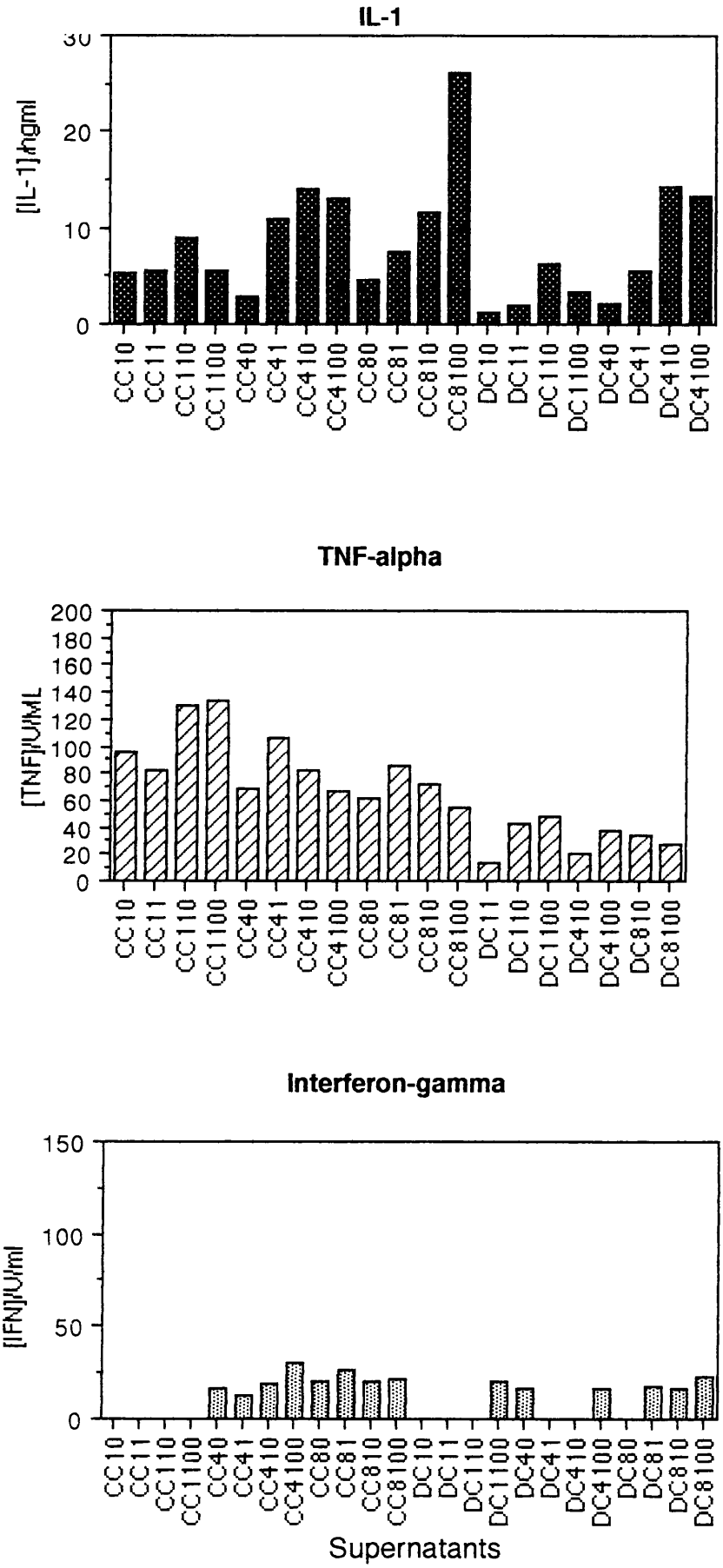
Figures 1a-1d: Show the concentrations of IL-1, TNF-α and IFN-γ in each of the IL-2 or anti-CD3 activated PBMC supernatants. Filled bars with white dots show results of IL-1 concentrations in $ngml^{-1}$. Striped bars show results of TNF-α concentrations in Units(U)/ml. Unfilled bars with black dots show results of IFN-γ concentrations in Units(U)/ml.

Figure 1a

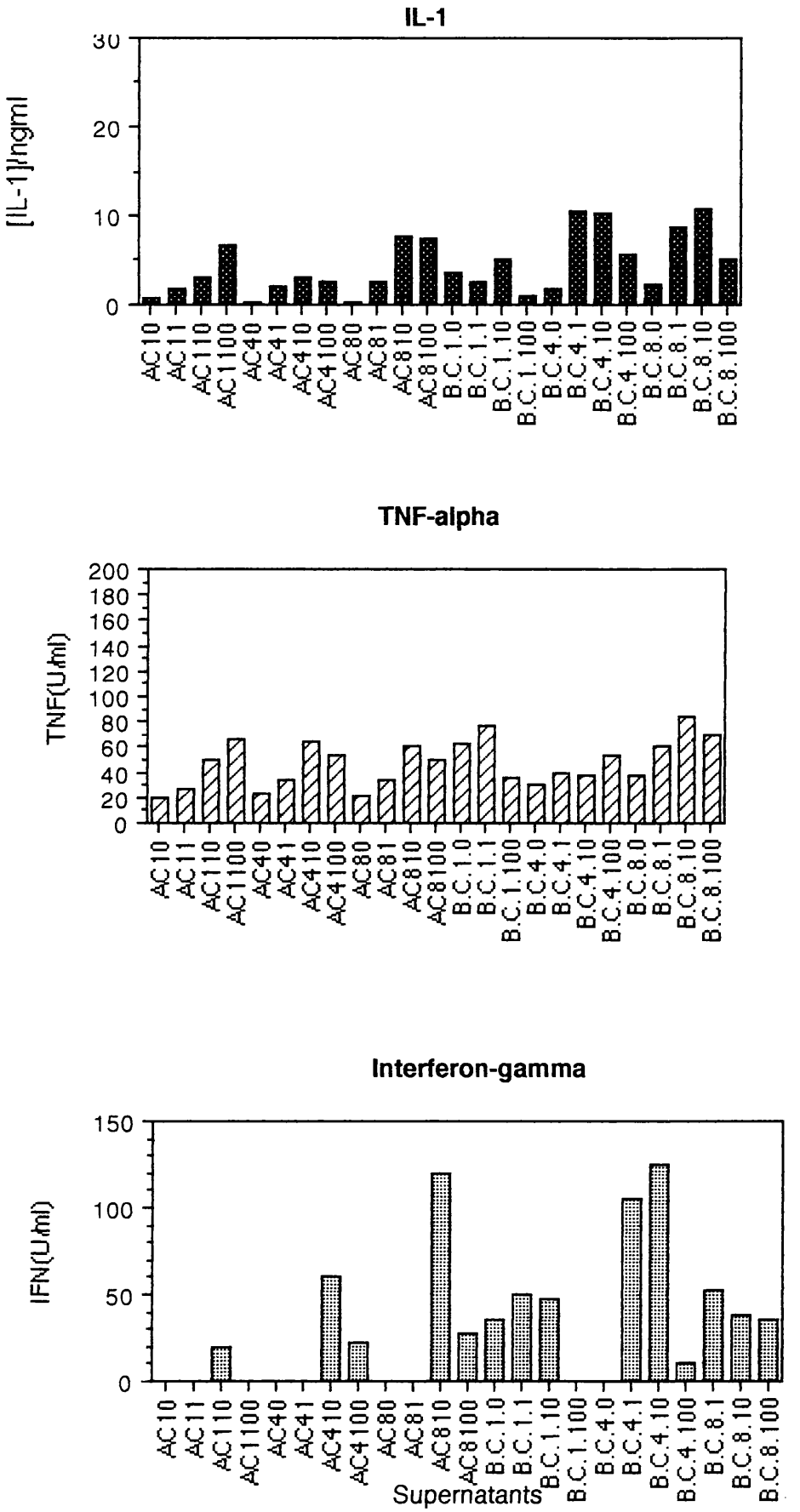
Graph showing cytokine concentrations present in each of the LAK supernatants (IL-2 stimulated: Donor A+B)



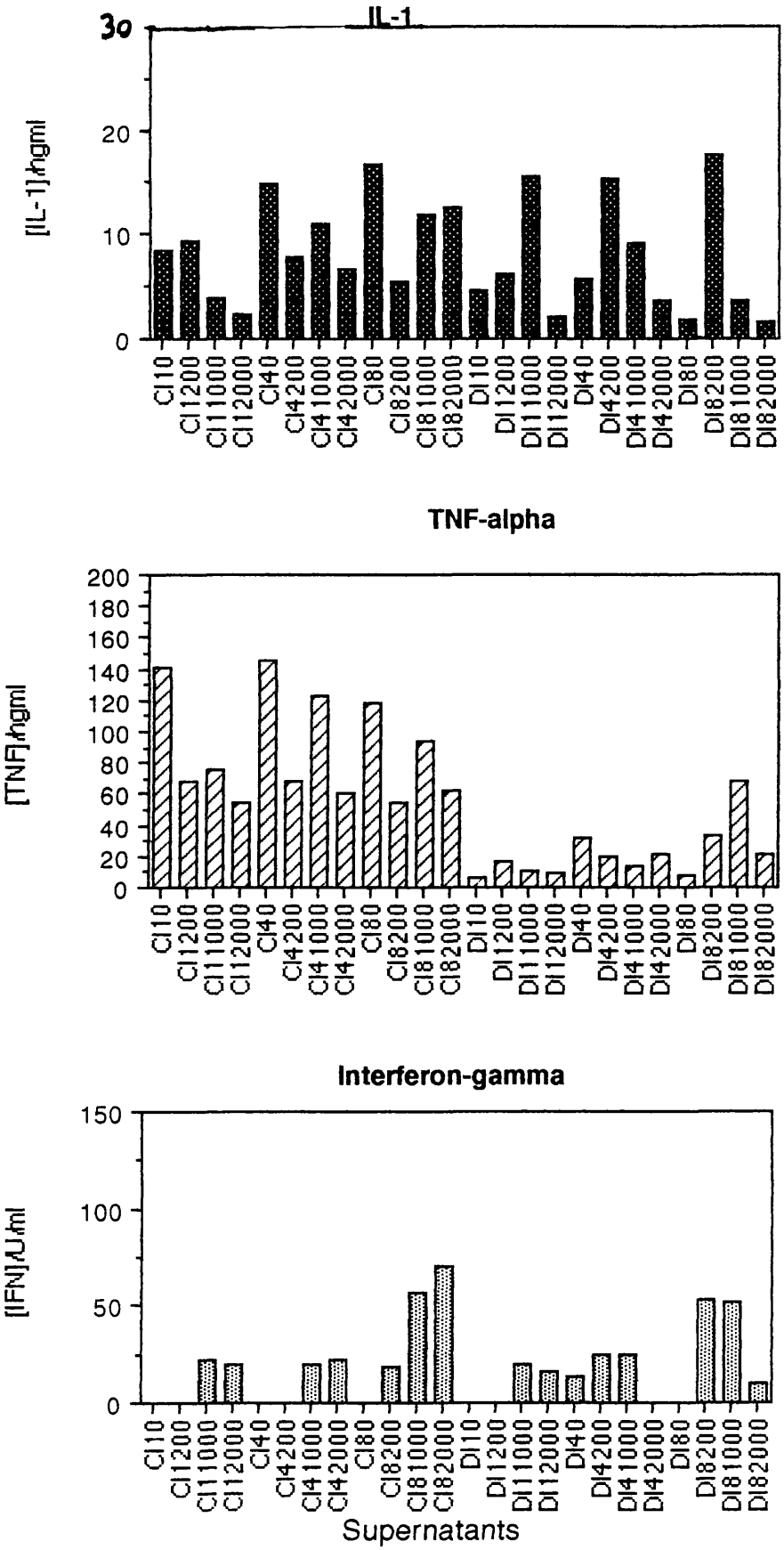
Graph showing cytokine concentrations present in each of the LAK supernatants (CD3 stimulated : Donors C+D)



Graph showing cytokine concentrations present in each of the LAK supernatants (CD3 stimulated : Donors A+B)



Graph showing cytokine concentrations present in each of the LAK supernatants (IL-2 stimulated : Donors C+D)



4.3 : Which monocyte separation technique to use?

Of the methods examined to separate monocytes from peripheral blood (i.e. density separation medium and adherence to plastics), a number of factors had to be considered before choosing one methodology which would be used in all of the ensuing experiments.
These factors were;

i) purity (examined using flow cytometry),
ii) yield (determined using a haemocytometer i.e.. /ml of whole blood),
iii) duration of method (hours)
iv) activation state (examined by clumping of the monocytes when observed under the microscope)

The monocytes obtained were observed taking each factor into consideration and the best technique was used to produce monocytes in all ensuing experiments. The blood used in all the experiments was kindly donated by members of the Surgery Department who were young and healthy. See Section 3.4 for methods. The cells were examined on the Coulter Epics, to determine purity, using antibodies specific to the different sub populations of white cells.

135ml of blood was obtained from a normal healthy volunteer from this :

45ml was used to determine the proportion of monocytes present in this volume of fresh peripheral blood. The total number of lymphoid cells were $46x10^6$. An aliquot of these cells were stained using monoclonal antibodies and examined using flow cytometry. From the data presented in Table 2, 36.5% of cells stained positive using the anti-CD14 antibody (mean of a triplicate) and Therefore the number of cells which were monocytes is 36.5% of $46x10^6$ which gives a figure of $16.8x10^6$

45ml was used in a monocyte separation using Nycodenz monocytes. Using this method, $5x10^6$ cells were recovered from 45ml of blood. An aliquot of the cells was again examined using monoclonal antibodies and flow cytometry, to determine monocyte purity. The % of CD14 positive cells was found to be 92.1%(mean of a triplicate)(see Table 2). Therefore the number monocytes obtained from 45ml of blood, , was 92.1% of $5x10^6$ which gives a figure of $4.6x10^6$ monocytes

45ml was used in a monocyte separation using the Adherence method. Using this method, $2x10^6$ cells were recovered from 45ml of blood. An aliquot of the cells was again examined using monoclonal antibodies and the flow cytometry, to determine monocyte purity. The % of CD14 positive cells was found to be 66.1%(mean of a triplicate)(see Table 2). Therefore the number monocytes obtained from 45ml of blood, upon calculation from the data, was 66.1% of $2x10^6$ which gives a figure of $1.3x10^6$ monocytes.

Therefore the Nycodenz monocyte method was the method giving the highest yield and purity but it was a much lengthier procedure and so I examined ways to improve the yield and purity of the adherence method.

4.3.1 : Adherence Method

The Adherence method of separation was faster to complete but did not produce a large yield of pure monocytes so the use of different brands of culture flasks and the %FCS in the culture media was varied to try and improve the adherence method outlined earlier. $110x10^6$ PBMC were obtained from 100ml of fresh venous blood donated by a healthy volunteer. $15x10^6$ PBMC were placed into each of the BIBBY and GRENIER flasks together with 15ml of RPMI 1640 (containing different amounts of FCS see Table 2). $30x10^6$ cells were placed into a FALCON T75 flask with 20ml of RPMI 1640. The cells were then incubated for 30 min see page .

As can be seen from the results shown in Table 3, the number of cells obtained was so low that an analysis of the monocyte purity could not be made. Therefore the slightly longer procedure using Nycodenz monocytes was adopted and used in all further experiments.

4.3.2 : Nycodenz Monocytes

The monocytes obtained using Nycodenz monocytes method of separation never produced a yield of 100% monocytes, as examined by flow cytometry, so the contaminating populations of cells must be noted as these cells could be involved in cytokine induced tumour cell cytotoxicity. Contaminating sub-populations were stained using specific antibodies versus the cells and observed using flow cytometry (See Section 3.4.1).

The examination of the purity of monocytes and the contaminating sub populations was constantly examined and monitored throughout the series of experiments. A typical result is shown in Table 4. It must be noted that after analysis contaminating B cells (which are non-adherent) were also removed by carefully washing the cells i.e. the adherent monocytes remained in the flask.

The Nycodenz monocytes method was used to obtain all of the monocytes in the ensuing experiments, as this method produced the best yields and purity of all the methods assessed.

Table 2

A table showing the results obtained after flow cytometry upon PBMC and monocytes after their relevant cell separations

Normal whole population	**% cells +ve**	**No. cells recovered**	**Monocyte yield/(cells)**
Cells	0		
Cells +FITC	1.1		
Cells+ A-CD14+FITC	36.5	$46x10^6$	$16.8x10^6$
Nycodenz Monocytes Method			
Cells	0.2		
Cells +FITC	0.8		
Cells+ A-CD14+FITC	92.1	$5x10^6$	$4.6 x10^6$
Adherence Method			
Cells	0.7		
Cells +FITC	4.0		
Cells+ A-CD14+FITC	66.1	$2x10^6$	$1.3x10^6$

Note A is used as an abbreviation for anti- when stating antibody type. This experiment was repeated with a different donor and similar results were obtained.

Table 3

A table showing the number of cells obtained/ flask after adherence

No. PBMC/ Flask ($x10^6$)	Flask Brand	% FCS	No. monocytes obtain ($x10^6$)
15	Greiner	10	0.3
15	Greiner	5	0.2
15	Bibby	...10	0.3
15	Bibby	5	0.1
30	Falcon	...10	1.2

Table 4

A table showing examples of monocyte purity and contaminating subpopulations after extraction using Nycodenz monocytes.

Cells plus Antibody	Cell type Stained	Range % Cells Stained
Cells	None	0 to 0.1
Cells + FITC	Background	1.4 to 2.1
Cells + A-CD14 + FITC	Monocyte	81.8 to 87.6
Cells + A-CD3 + FITC	T cell	4.1 to 9.1
Cells + A-CD19 + FITC	B cell	0.9 to 10.0
Cells + NKH-1 + FITC	NK cell	60.9 to 5.0

4.4 : What effect do the LAK cell supernatants have on monocyte tumour cytotoxicity as determined by the monocyte cytotoxicity assay

The LAK supernatants produced earlier were then used to try and enhance peripheral blood monocyte (separated from whole blood using Nycodenz monocytes) tumour cytotoxicity against target cells SW742, in vitro. This was analysed using a 48hr radioimmunoassay pre-labelling the SW742 cells with ^{3}H-uridine.

Cells from the monocyte-like cell line U937 were also used as effector cells (instead of monocytes), for two distinct reasons. Firstly, to show that any effect the supernatants had upon the effector cell population was a genuine response which could be duplicated and not an artefact from the variation in monocyte donor and secondly because of the limitations of blood donation in the laboratory. As can be seen from graphs 2a-2d the monocytes in general behave in an identical manner with regards to cytotoxicity as do the U937 cell line.

Figure 2a is of three graphs depicting the cytotoxic effect supernatants A (IL-2 stimulated) had on peripheral blood monocytes and U937 effector cells (a monocytic cell line) upon the tumour target cells SW742. As can be seen from the graphs the U937 cell line is affected in a similar way to peripheral blood monocytes. A general trend can be seen in both graphs but intra assay variation is still present and the ability of the target cells to take up ^{3}H-Uridine varied in each ease. Nevertheless for donor A the 200 and 2000 Unit doses bring about heightened cytotoxicity with maximal cytotoxicity being reached after about four days of incubation with IL-2.

With donor B, shown in **figure 2b**, again maximal stimulation was seen after four days with the 200Units of IL-2 being the optimal dose. **Figure 2c** shows the cytotoxic profiles of effector cells stimulated with supernatants from donor C (IL-2 stimulated). For donor C there tends to be high basal cytotoxicity and this remains in general unaffected by addition of supernatants from PBMC stimulated with increasing amounts of IL-2. **Figure 2d** shows optimal tumour cytotoxicity when the cells are stimulated with 200 Units per ml of IL-2, maximal cytotoxicity being reached at day 4.

Figure 3a and 3b depicts the cytotoxic profiles of the effector cells stimulated with supernatants from donor A and B respectively (anti-CD3 stimulated). There appeared to be no real trend with the mean results being the same (no matter except that the supernatants produced from unstimulated cells achieved a higher % generated cytotoxicity than those

produced by stimulated supernatants. For donor C, **figure 3c**, there tends to be no stimulation caused by the anti-CD3 supernatants. Cytotoxicities corresponding to those determined with unstimulated PBMC supernatants are seen across the board. However the general level of cytotoxicity can be seen to be higher than those cytotoxicities observed by the other donors supernatants. For donor D , there again seems to be no real trend with in general the highest cytotoxicities seen when the supernatant's used have been unstimulated with anti-CD3, **figure3d**. The results depicted in figure 2a-2d and 3a-3d are all the data I was able to amass due to the time constraints of the project. Further experiments would need to be carried out to verify there statistical and reproducible content.

Figures 2a-2d: Shows the % cytotoxicity generated by the cytokine rich supernatants, derived from IL-2 induced PBMC, upon peripheral blood monocytes, as seen using a 48hr ^{3}H-Uridine release assay. Figures a-d represent the four donors, with each of the three graphs per figure representing the different initial time the PBMC were incubated with increasing concentrations of IL-2 (x-axis) i.e. 0, 200, 1000, 2000 units per/ml containing $1*10^{6}$ PBMC.

U = Triangle shape =The U937 cell line were used as effector cells.

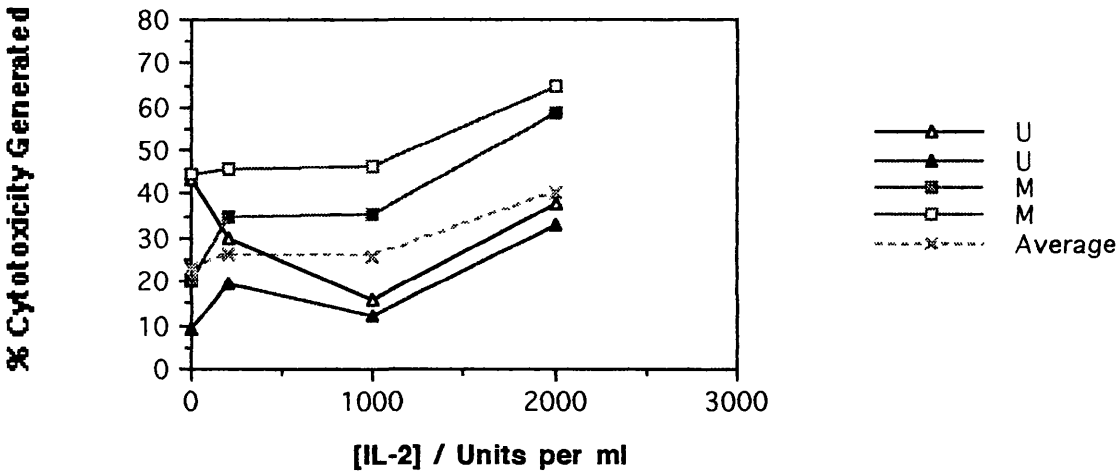
M = Square shape = Monocytes were used as effector cells

The filled and unfilled shapes represent data obtained in diiferent separate experiments, with monocytes harvested from different donors.

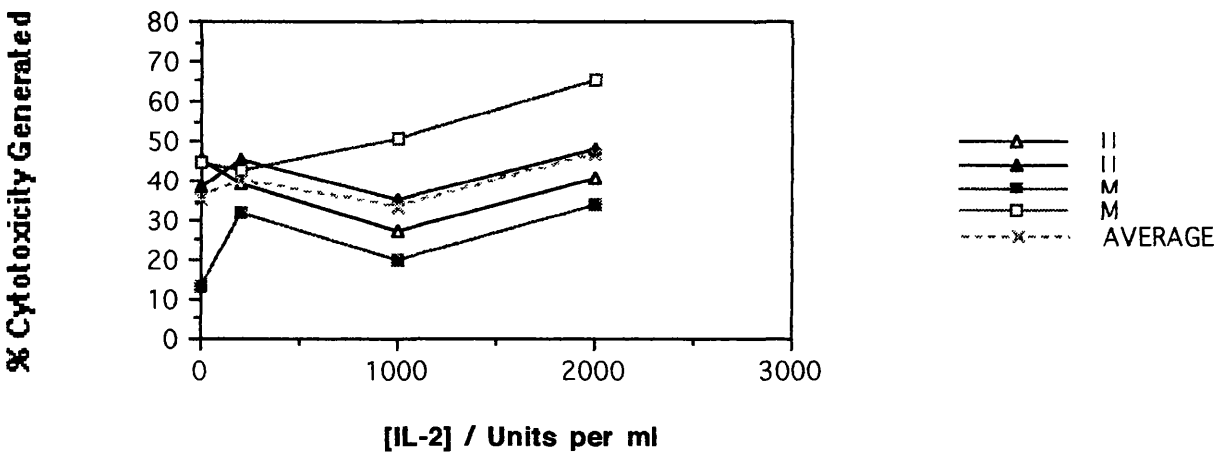
---x--- = The mean result of the data points per IL-2 dose

% Cytotoxicity generated by cytokine supernatants, derived from donor A PBMC, upon peripheral blood monocytes.

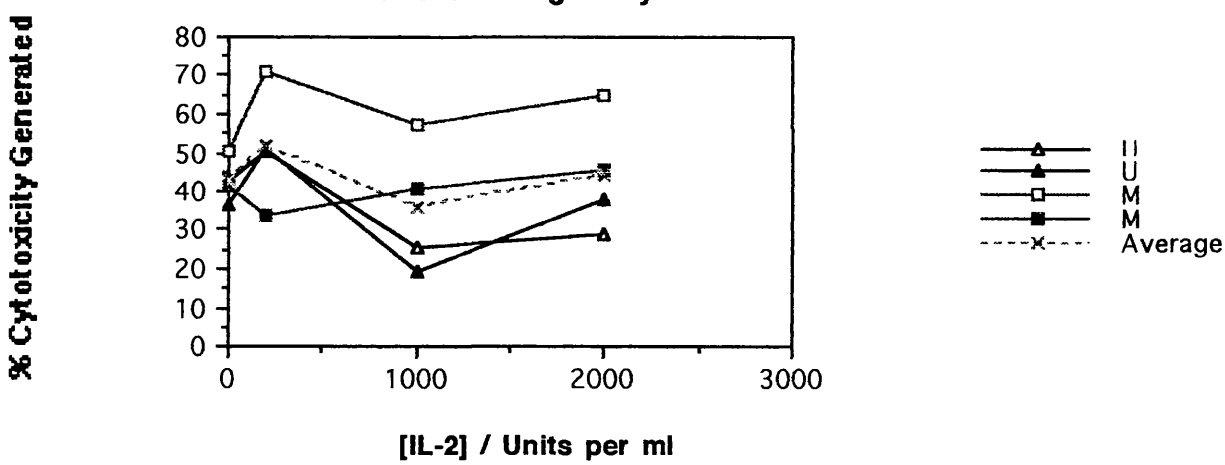
IL-2 induced for one day.



IL-2 induced for four days.

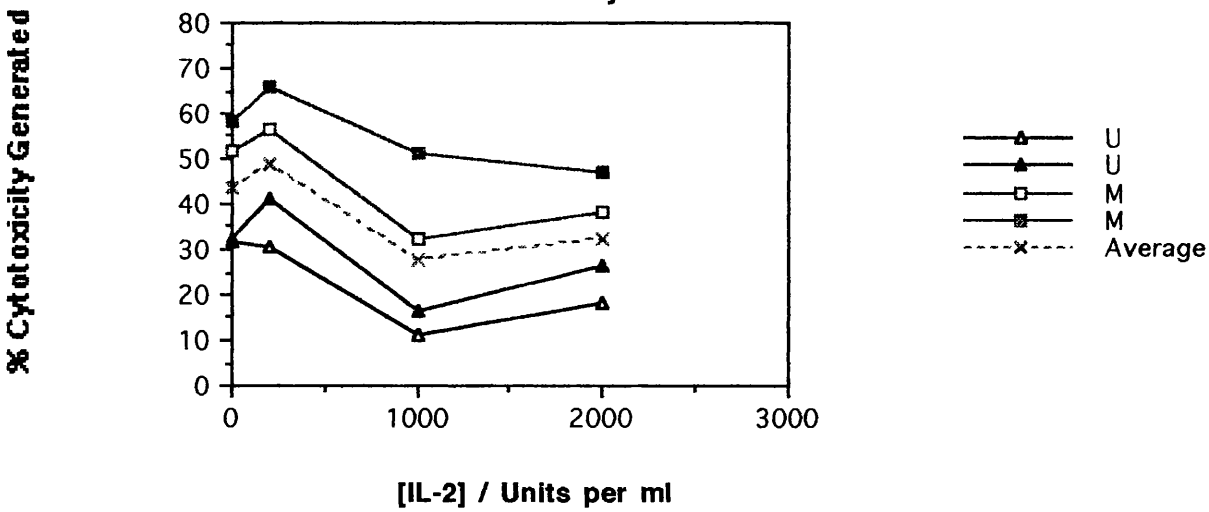


IL-2 induced for eight days.

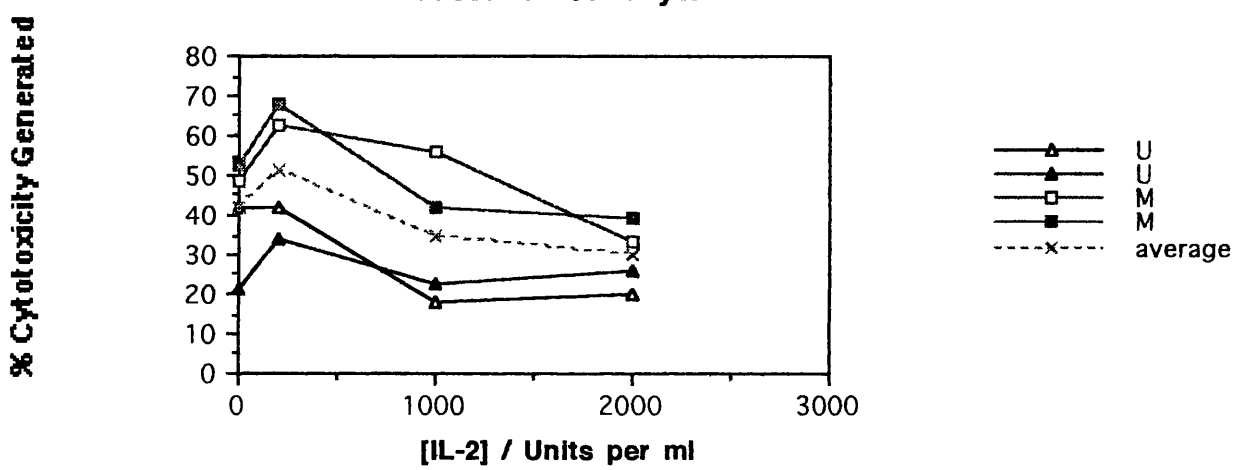


% Cytotoxicity generated by cytokine supernatants, derived from donor B PBMC, upon peripheral blood monocytes.

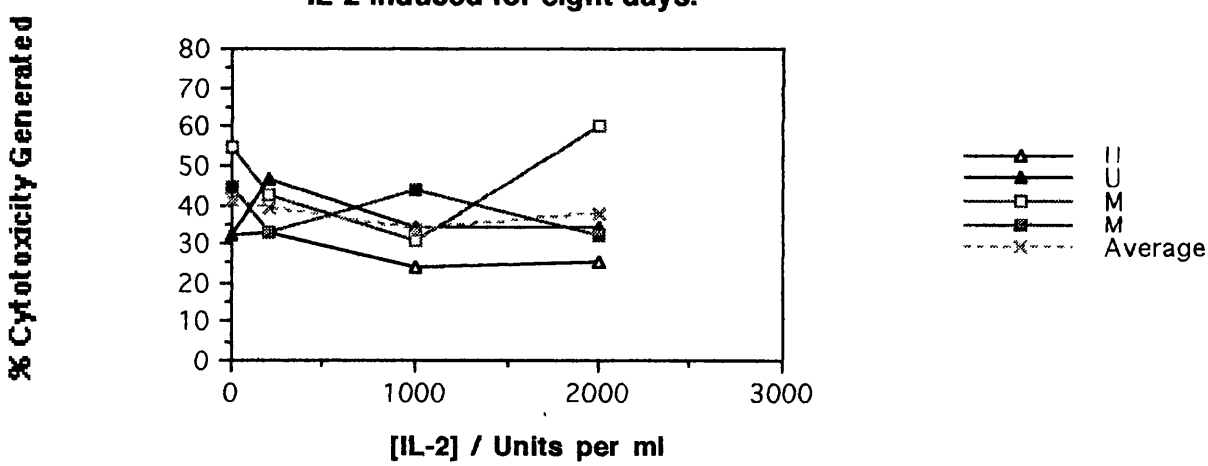
IL-2 induced for one day.



IL-2 induced for four days.

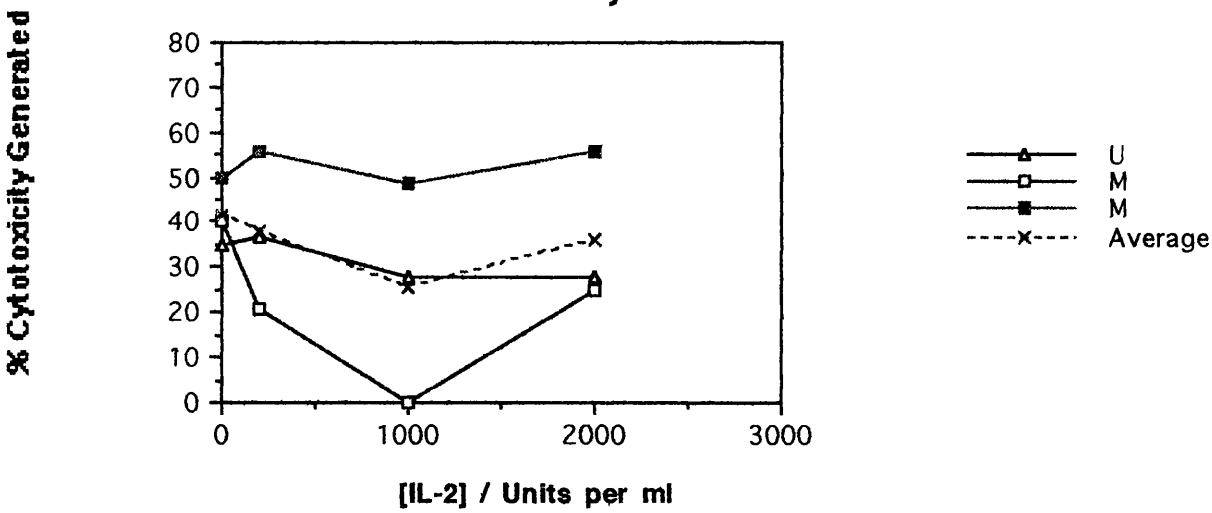


IL-2 induced for eight days.

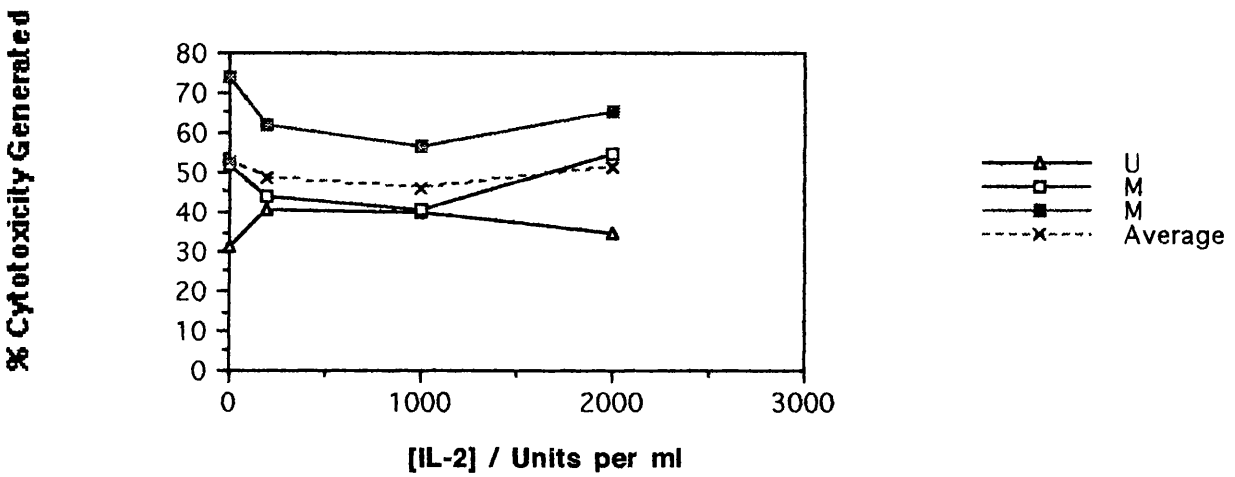


% Cytotoxicity generated by cytokine supernatants, derived from donor C PBMC, upon peripheral blood monocytes.

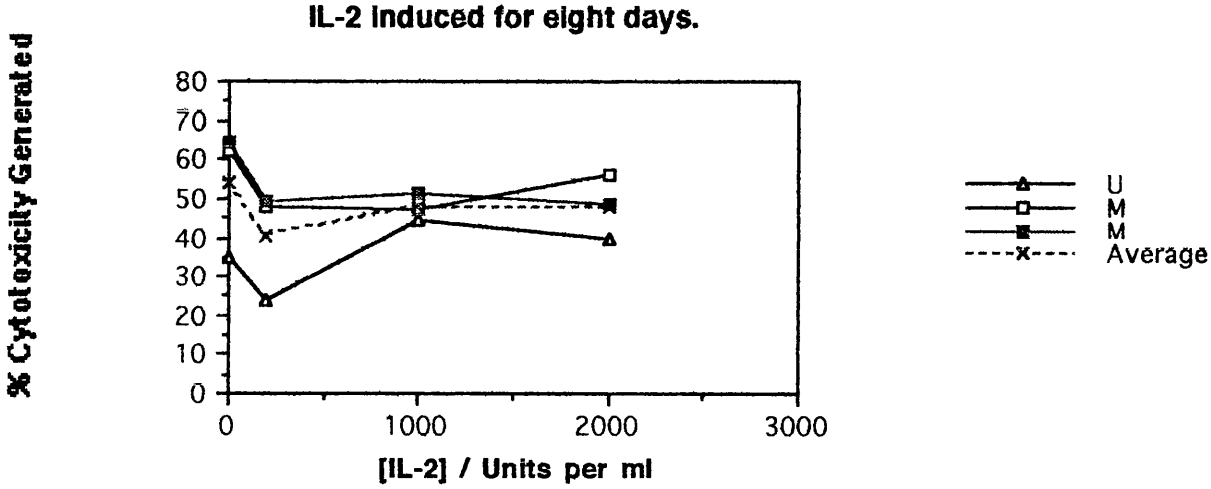
IL-2 induced for one day.



IL-2 induced for four days

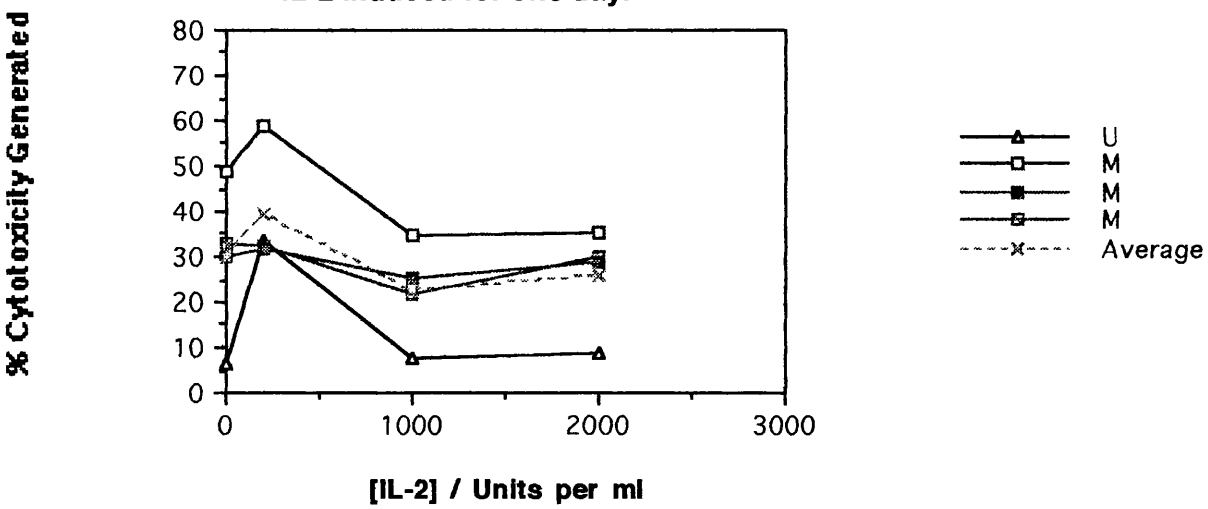


IL-2 induced for eight days.

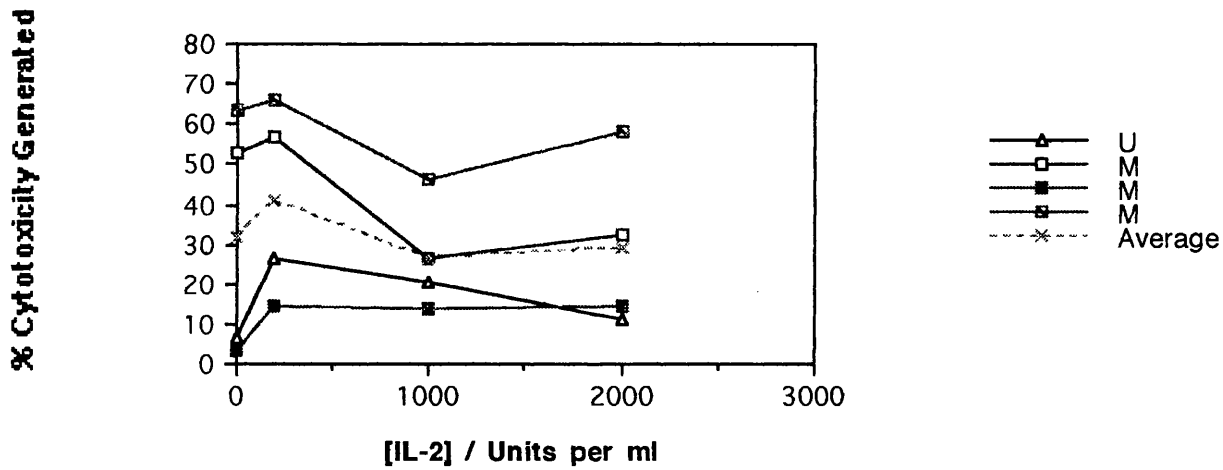


% Cytotoxicity generated by cytokine supernatants, derived from donor D PBMC, upon peripheral blood monocytes.

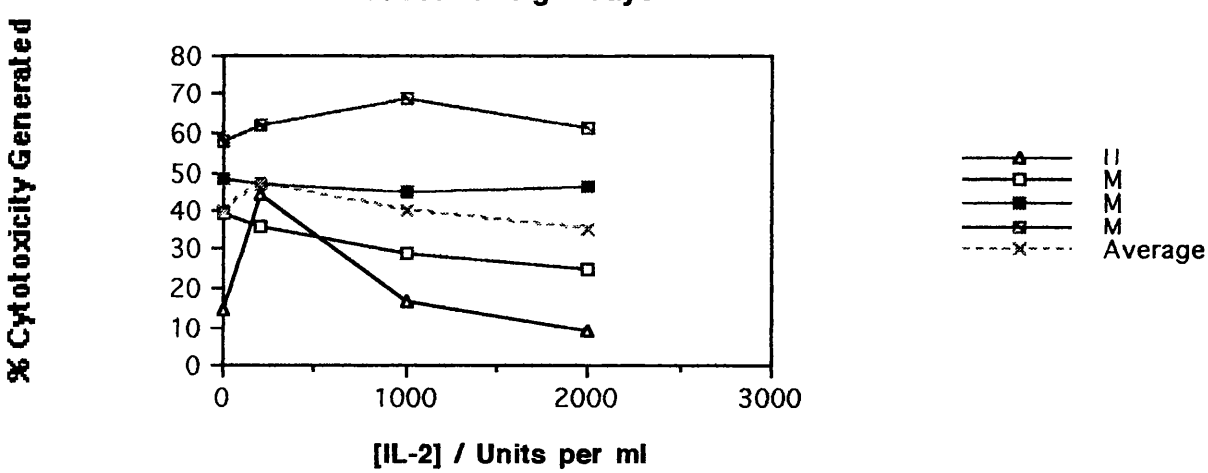
IL-2 induced for one day.



IL-2 induced for four days.



IL-2 induced for eight days.



Figures 3a-3d: Shows the % cytotoxicity generated by the cytokine rich supernatants, derived from anti-CD3 antibody induced PBMC, upon peripheral blood monocytes, as seen using a 48hr ^{3}H-Uridine release assay. Figures a-d represent the four donors, with each of the three graphs per figure representing the different initial time the PBMC were incubated with increasing concentrations of anti-CD3 antibody (x-axis) i.e. 0, 1, 10, 100 ng/ml containing $1*10^{6}$ PBMC.

U = Triangle shape =The U937 cell line were used as effector cells.

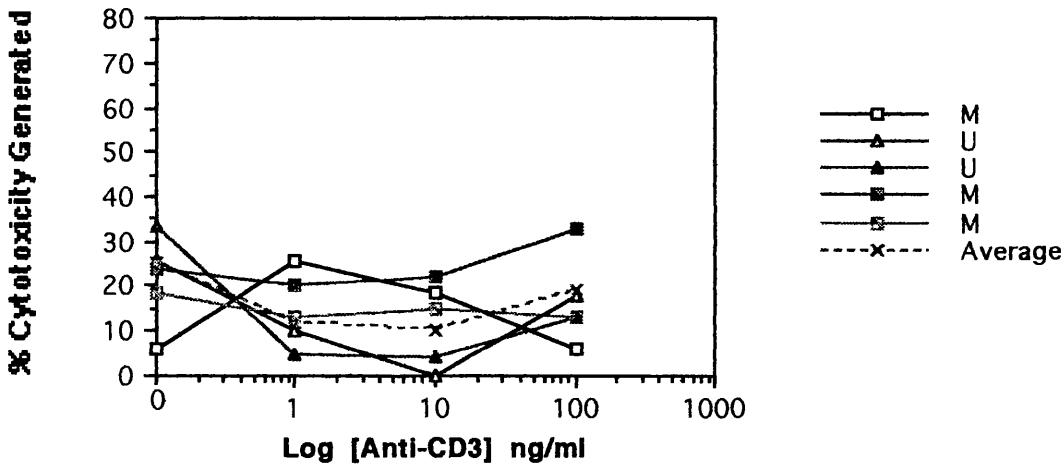
M = Square shape = Monocytes were used as effector cells

The filled and unfilled shapes represent data obtained in diiferent separate experiments, with monocytes harvested from different donors.

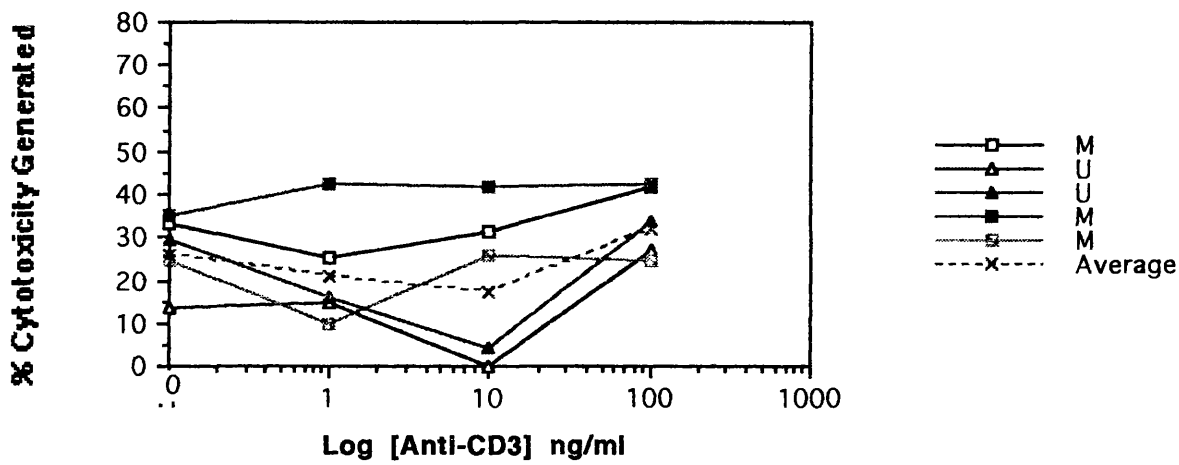
---x--- = The mean result of the data points per anti-CD3 dose

% Cytotoxicity generated by cytokine supernatants, derived from donor A PBMC, upon peripheral blood monocytes.

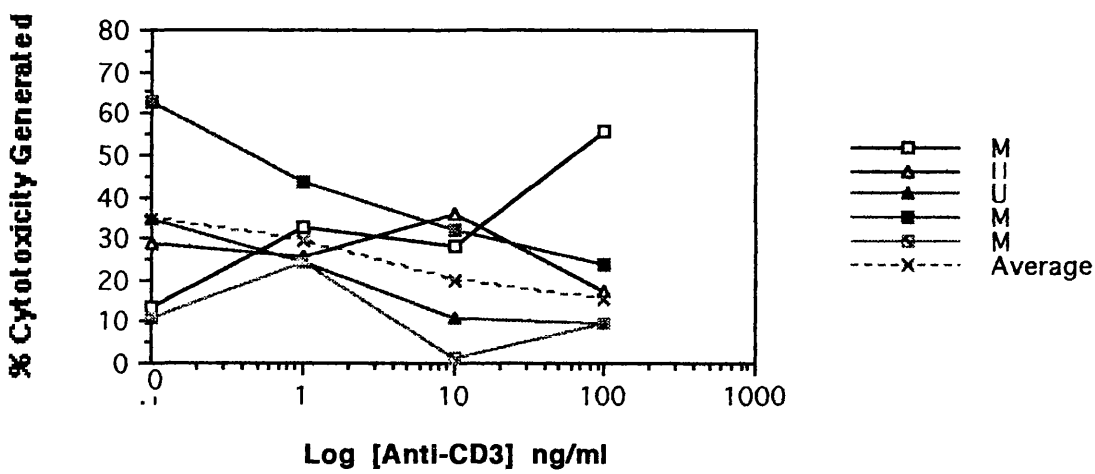
Anti-CD3 induced for one day.



Anti-CD3 induced for four days.

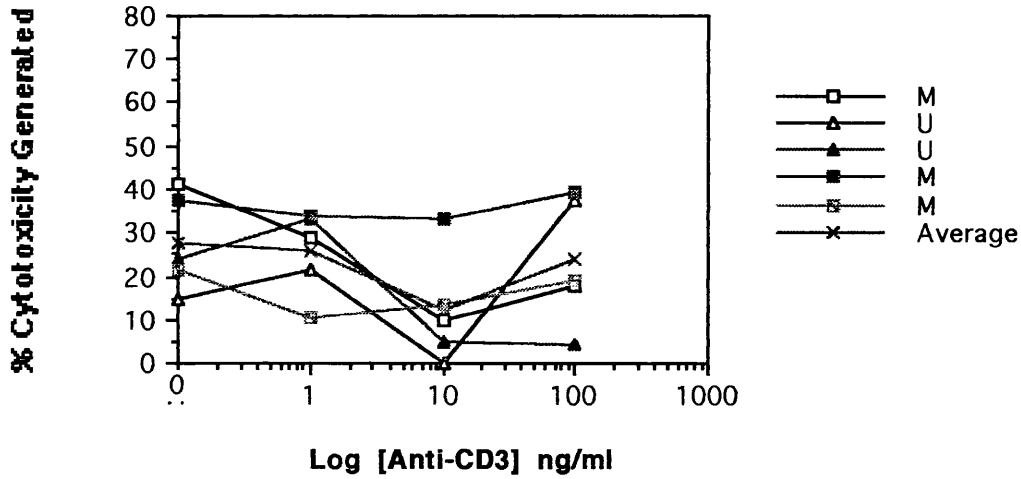


Anti-CD3 induced for eight days.

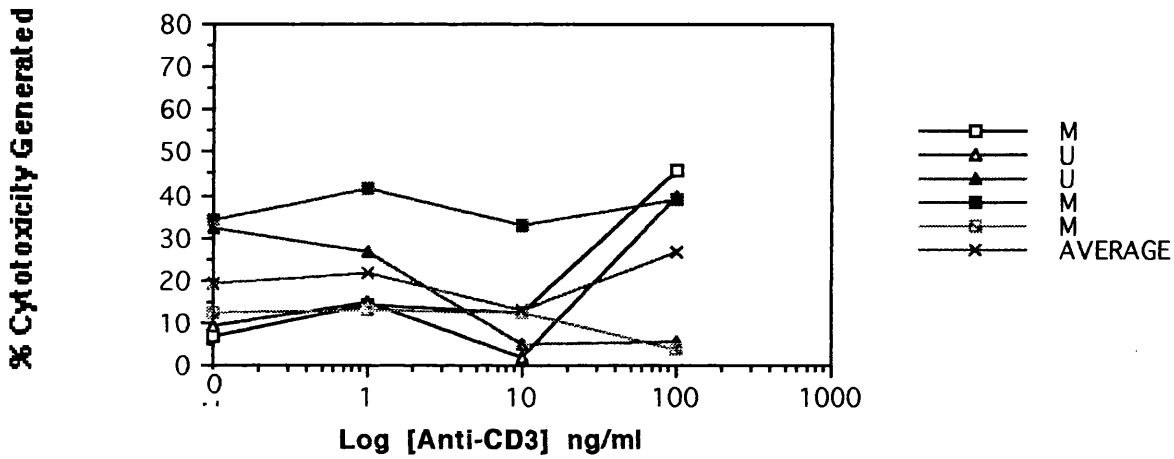


% Cytotoxicity generated by cytokine supernatants, derived from donor B PBMC, upon peripheral blood monocytes.

Anti-CD3 induced for one day.



Anti-CD3 induced for four days.



Anti-CD3 induced for eight days.

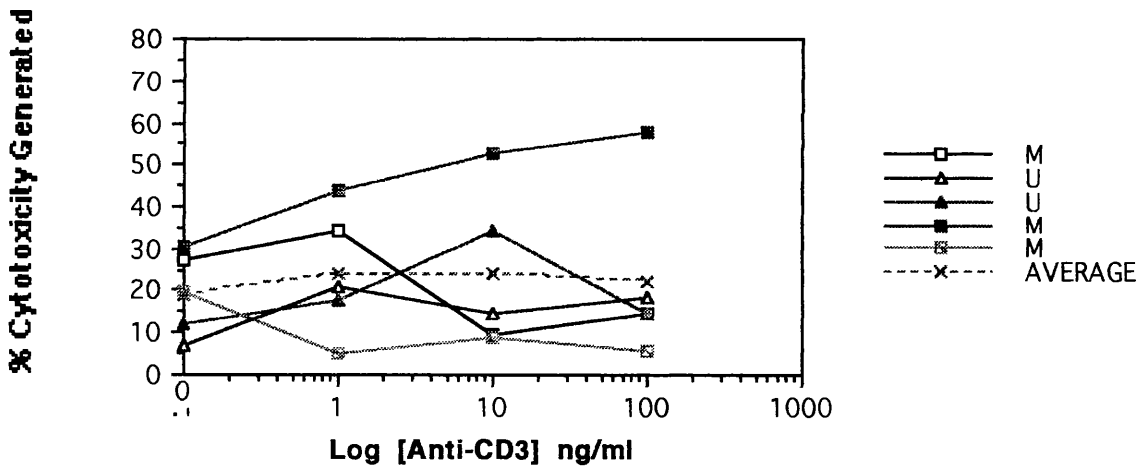
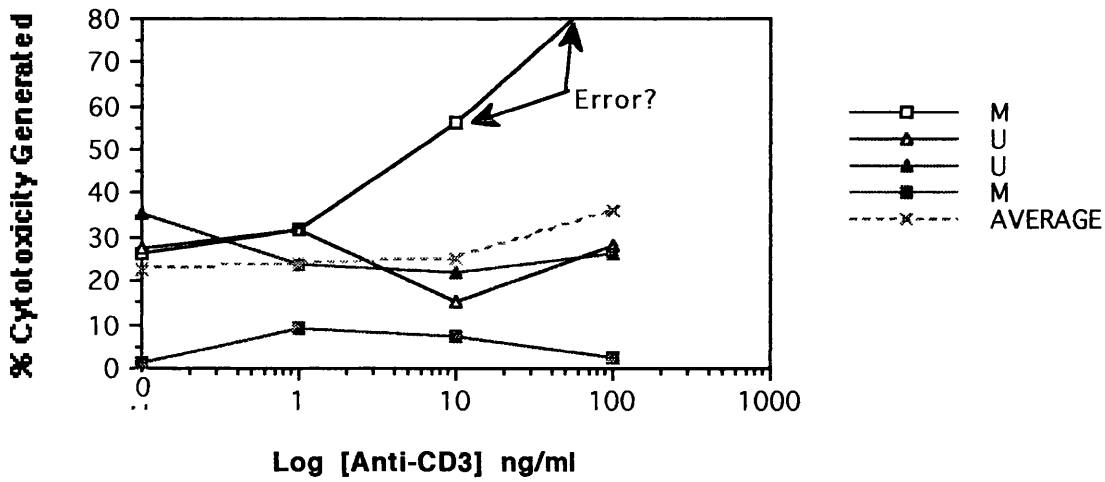


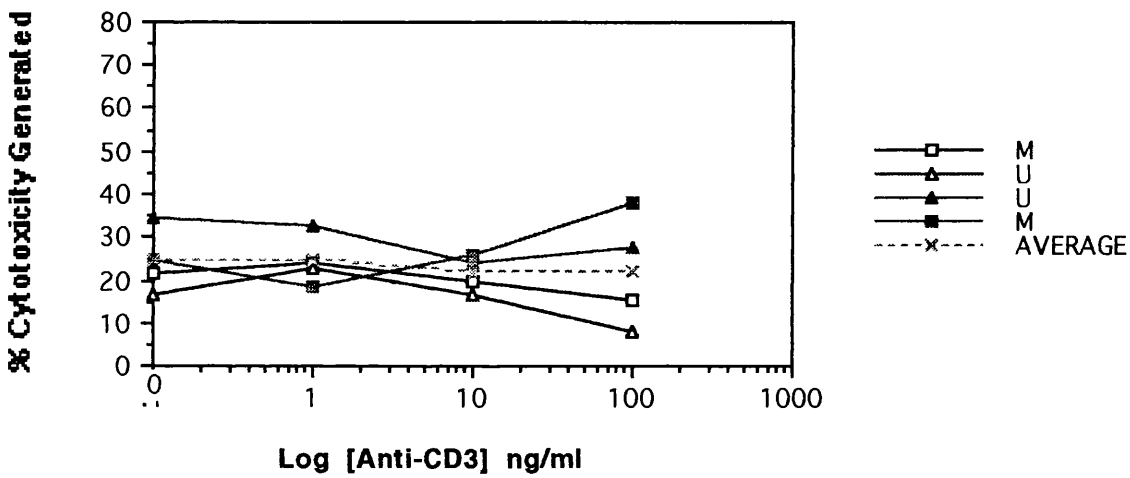
Figure 3c

% Cytotoxicity generated by cytokine supernatants, derived from donor C PBMC, upon peripheral blood monocytes.

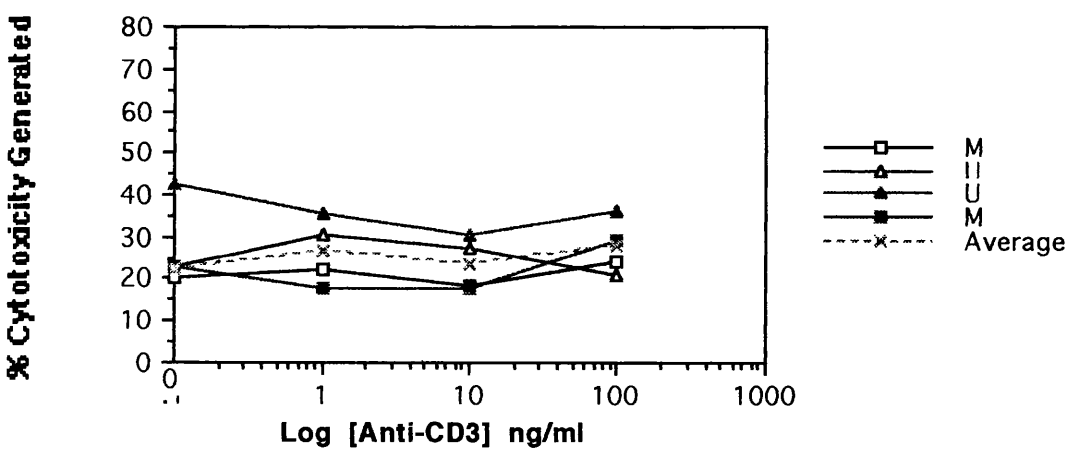
Anti-CD3 induced for one day.



Anti-CD3 induced for four days.

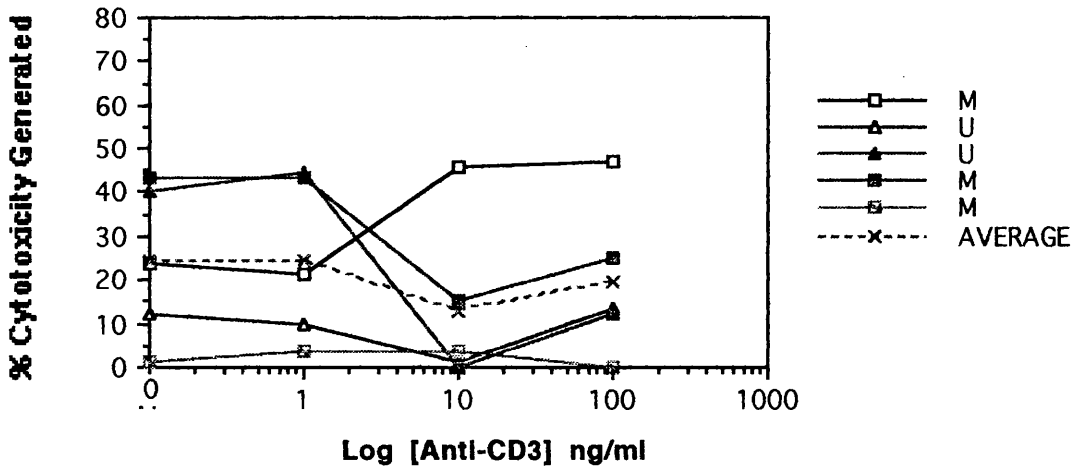


Anti-CD3 induced for eight days.

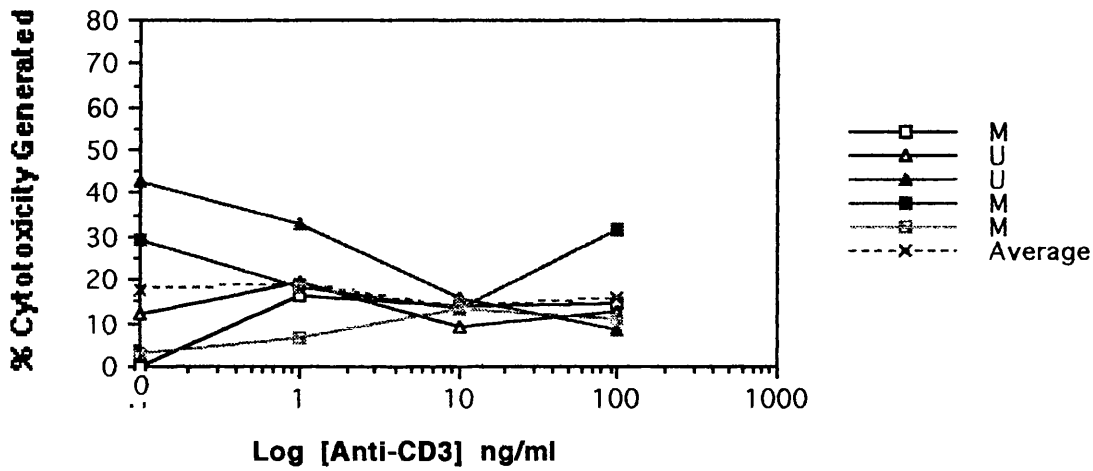


% Cytotoxicity generated by cytokine supernatants, derived from donor D PBMC, upon peripheral blood monocytes.

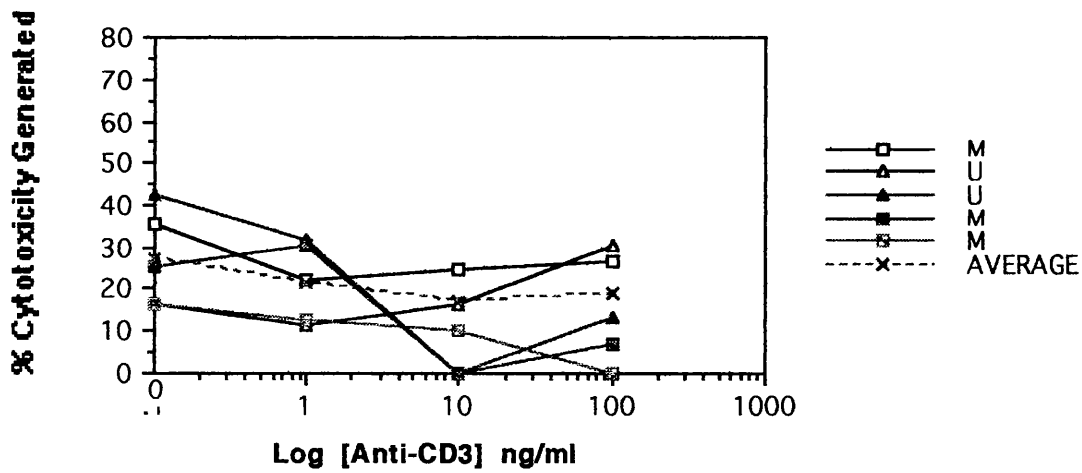
Anti-CD3 induced for one day.



Anti-CD3 induced for four days.



Anti-CD3 induced for eight days.



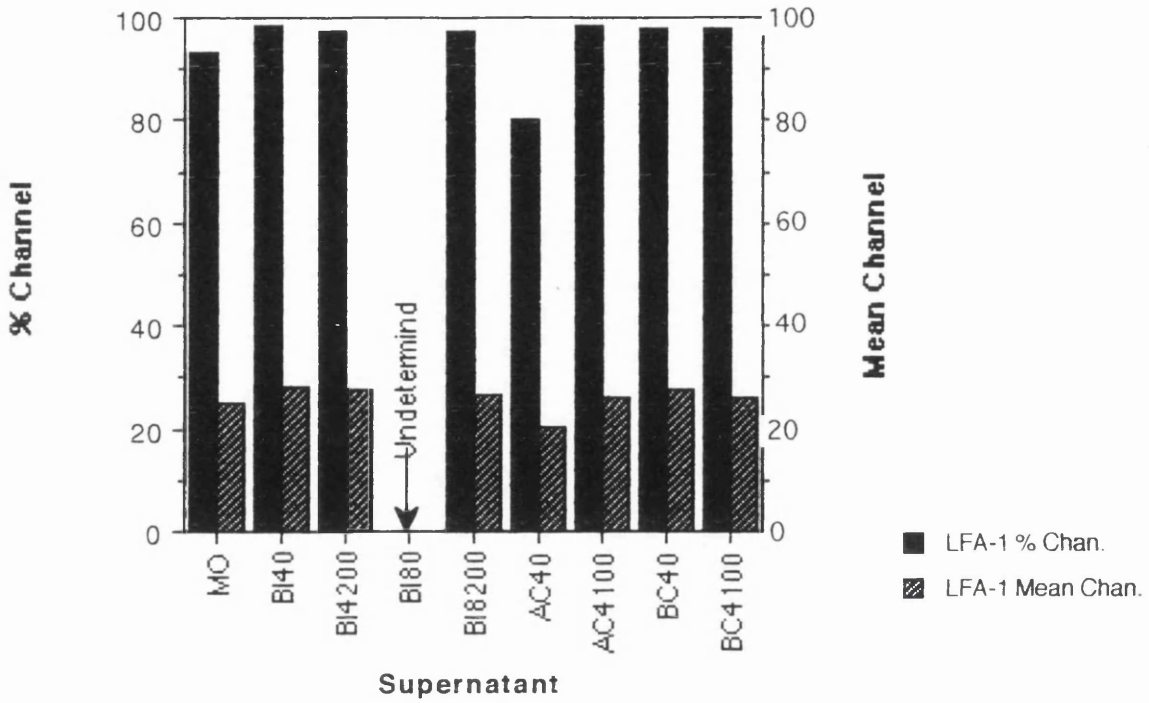
4.5 : What role do adhesion molecules play in monocyte tumour cytotoxicity

The activation of the monocytes by the LAK supernatants to become cytotoxic against SW742 cells may require cell to cell contact between the activated monocyte and the tumour cell. The upregulation of two adhesion molecules present on the monocyte were examined to consider this. The adhesion molecules in question were LFA-1 and Mac-1. The presence and level of these adhesion molecules was examined, using mouse anti human antibodies against LFA-1 and Mac-1, and detected using flow cytometry (see Section 3.3). Monocytes were examined pre and post LAK cell supernatant incubation, with the incubation time being similar to that used in the cytotoxicity assay. The upregulation of MHC II on the cell surface of the monocytes was also examined, in conjunction with addition of LAK supernatant. This was carried out to ascertain if LAK cells were upregulating monocyte antigen presenting capabilities.

After this had been evaluated anti-cytokine antisera was added to the supernatants prior to the incubation with monocytes. The cells were then examined using flow cytometry to establish differences in receptor profiles from those previously stated. The results highlighted a possible link between a specific cytokine induced upregulation of adhesion molecules and the induction of monocyte cytotoxicity against SW742 cells. The concentration of IL-2 would then be known which optimally promotes LAK cells to release cytokine/s, in the correct concentration, to activate monocyte tumour cell killing in vivo.

Figure 4 depicts the % Channel and Mean Channel, observed using the EPICS flow cytometer, for the monocyte cell surface adhesion molecules LFA-I and Mac-I (see Section 3.3), with and without certain of the LAK supernatants. As can be seen the number of each of the adhesion receptors upon each monocyte is directly proportional to the number of cells stained. There appeared to be no great change in the expression of LFA-1 on addition of the supernatants from that of unstimulated monocytes (MO). The Mac-I results showed a heightened expression per cell (% channel) and in the population (mean channel), when compared with the control, with those monocytes which had been pre-stimulated with supernatants produced from unstimulated LAK cells (i.e. W.X.Y.Z see section 4.1). Note that the supernatant B.I.1.0 experiment with LFA-1 did not work due a technical fault with the flow cytometer and thus no data is expressed for this. However the **figure 4** contains this experiment for completeness, so easy comparison can be made between the LFA-1 and Mac-1 data.

A GRAPH SHOWING LFA-1 % CHANNEL AND MEAN CHANNEL ON MONOCYTES STIMULATED WITH VARIOUS LAK SUPERNATANTS



B GRAPH SHOWING Mac-1 % CHANNEL AND MEAN CHANNEL ON MONOCYTES STIMULATED WITH VARIOUS LAK SUPERNATANTS

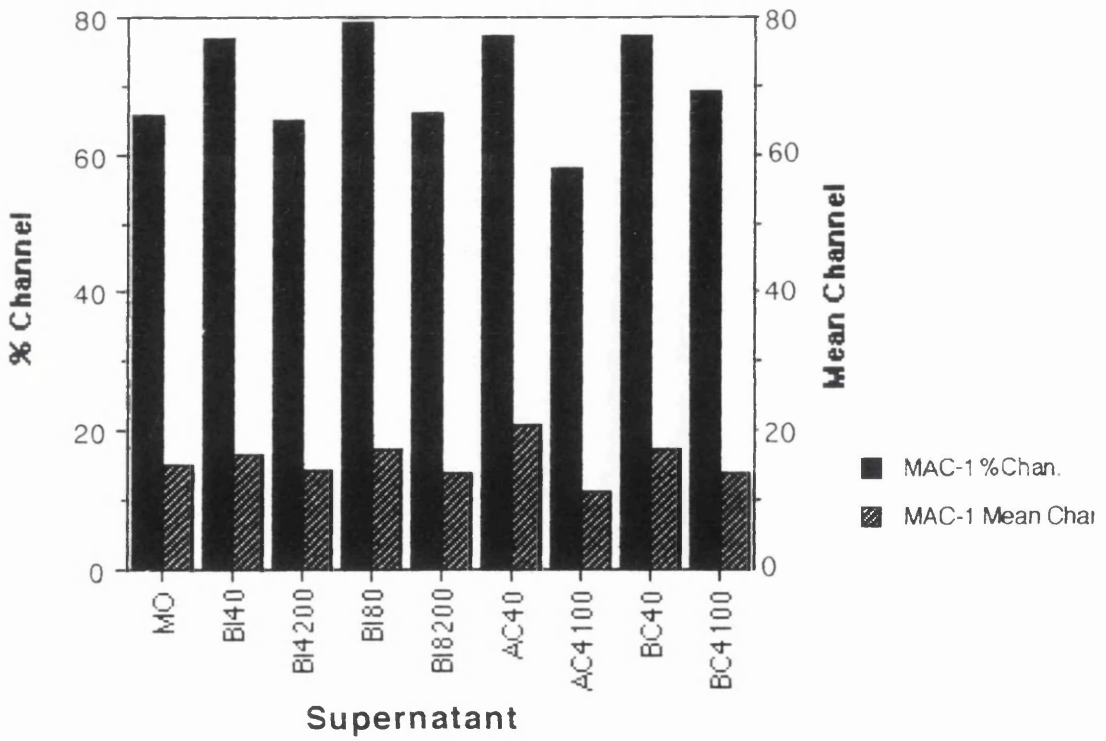


Figure 5a shows the presence of MHC II, on the monocyte population as a whole, when stimulated with certain of the LAK supernatants. On the whole there is not much change in MHC II expression over the whole range of supernatants, with approximately 90% of monocytes expressing the minimumn number of MHC II molecules dectable by the flow cytometer. However as can be seen from the graph the number of molecules per cell (Mean Channel) does increase on addition of particular LAK supernatants.

Figure 5a

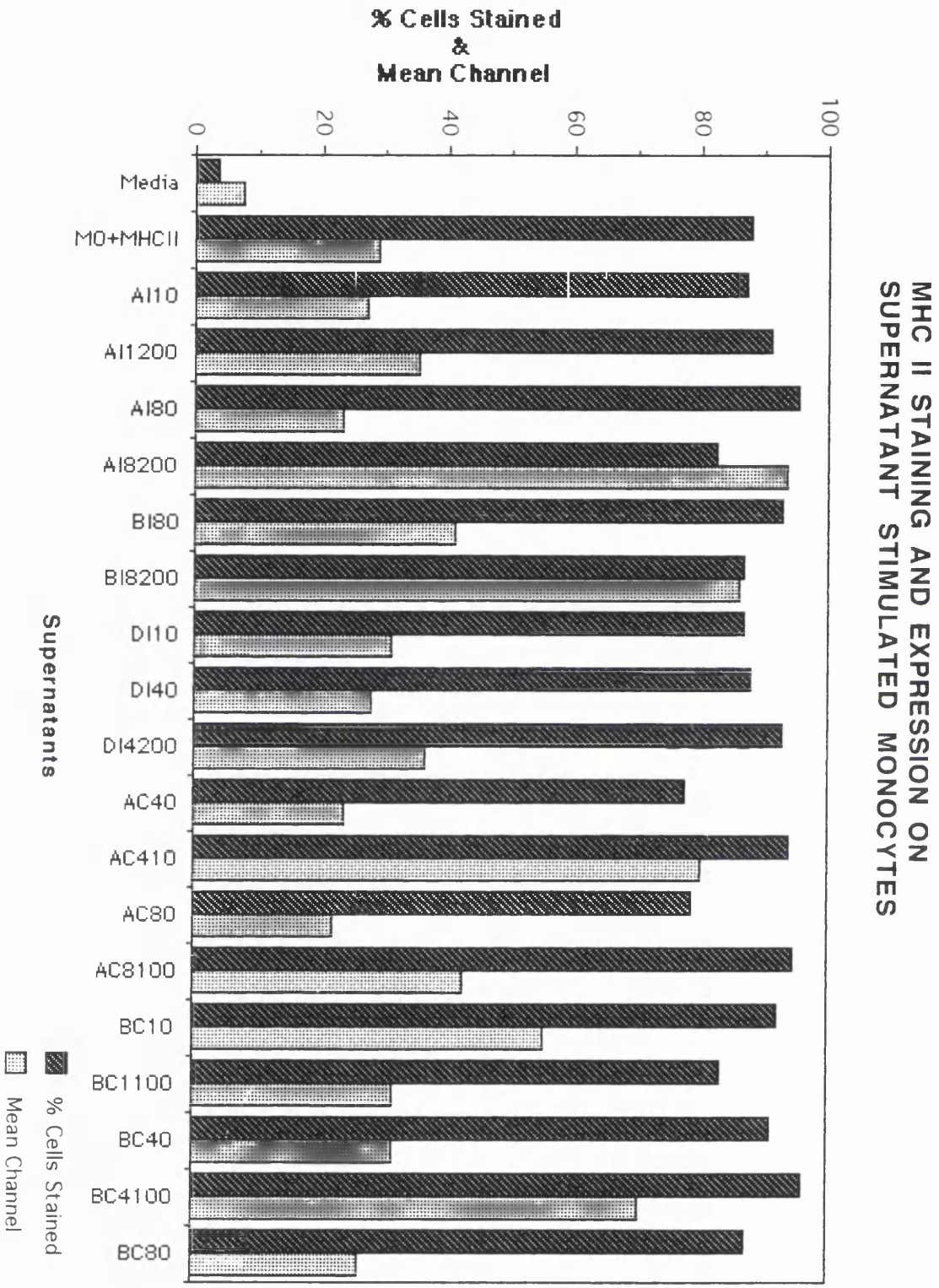
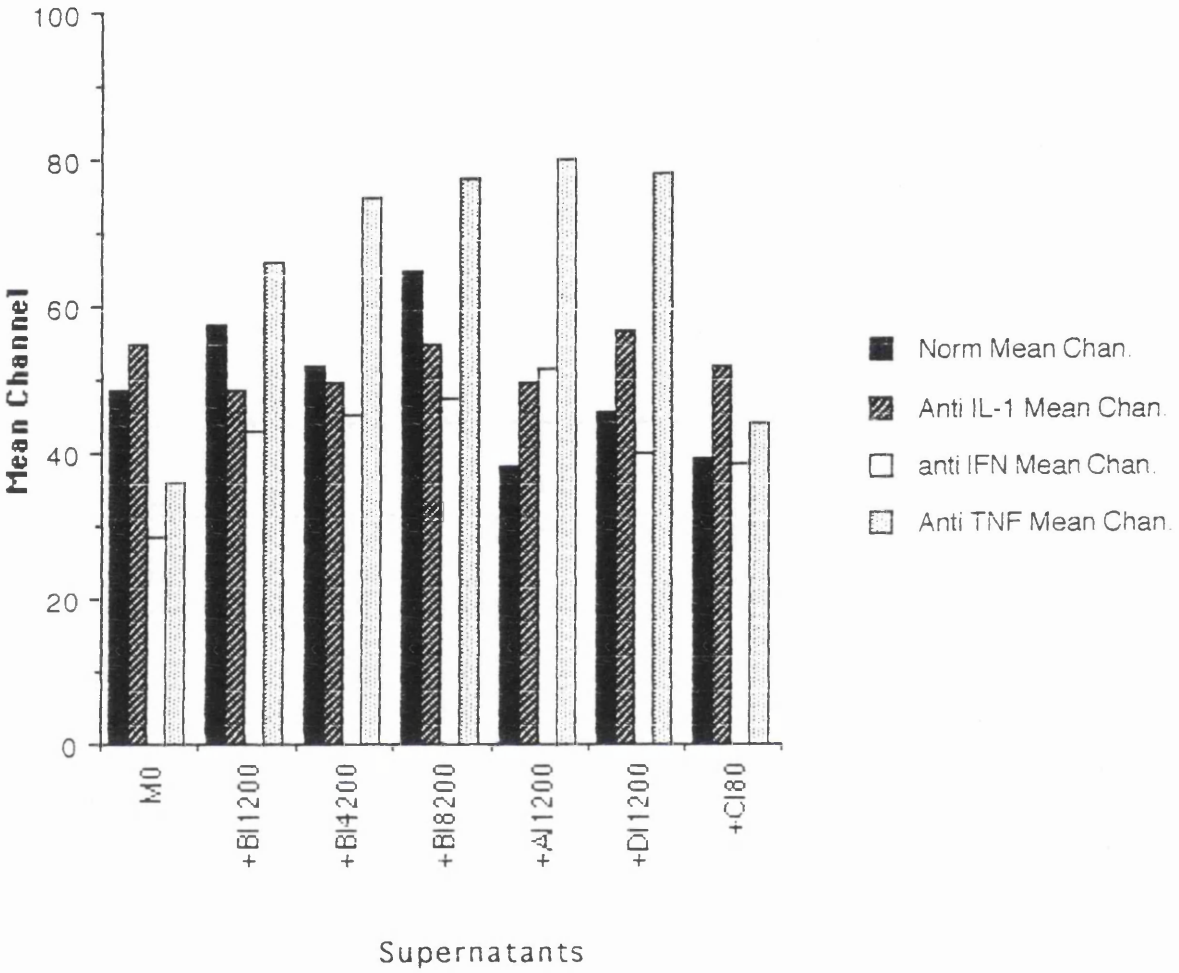

MHC II STAINING AND EXPRESSION ON SUPERNATANT STIMULATED MONOCYTES
% Cells Stained & Mean Channel
0
20
40
60
80
100
Media
M0+MHCII
AI10
AI1200
AI80
AI8200
BI80
BI8200
DI10
DI40
DI4200
AC40
AC410
AC80
AC8100
BC10
BC1100
BC40
BC4100
BC80
Supernatants
% Cells Stained
Mean Channel

Figure 5b depicts the density of MHC II on monocytes with at leatst one receptor when incubated with certain of the LAK supernatants, with or without neutralising antisera. For the donor B LAK supernatants removal of IL-1 and IFN-γ brought about a decrease in the density of MHCII per cell. TNF-alpha antisera when added to the LAK supernatants on the whole caused an increase in MHC II expression on the monocytes which were unstimulated, thus the antibody itself was probably causing this change i.e. throgh Fc binding.

Graph showing variations in MHC II mean channel readings on monocytes pre-incubated with LAK supernatants (and/or cytokine antisera)



4.6 : How do the activated monocytes destroy tumour cells?

The final set of experiments was designed to examine the mode of killing used by the monocytes to destroy the tumour target cells. Monocytes have several means of destroying tumour cells (see Section 1.6). but the primary cytokine used is TNF-alpha. Monocyte secretion of TNF-alpha was therefore analysed both pre and post stimulation with LAK supernatant(67).

Monocyte cytotoxicity experiments were carried out (seeSection 3.5) by incubating LAK cell supernatants with peripheral blood monocytes and then adding these activated cells to irradiated SW742 (with tritiated Uridine) and detecting killing in the usual way (see method). However before adding the Triton-X-100 the supernatants from each well were removed and stored. At a later date TNF ELISA's were carried out on these supernatants, to determine the concentration of TNF-alpha in each of the supernatants. This was carried out to examine a possible correlation between % cytotoxicity generated by the LAK supernatants and TNF-alpha secretion (a cytokine known to be associated with cell death; (54)). **Figure 6** shows a scatter graph of the TNF-alpha secreted by monocytes from a single donor when stimulated with different LAK supernatants against the % Cytotoxicity generated by these monocytes upon SW742 tumour target cells. The statisitics using a Spearman Rank Correlation show TNF-alpha secretion is very significantly correlated to % cytotoxicity generated ($r=0.6095$, $p=0.002$)

Note that the important factor here is that the monocytes were washed before addition of the tumour target cells, so none of the TNF-alpha present could be from the LAK supernatants.

Next, similar experiments were carried out to those above but this time adding neutralising antibodies against the cytokines IL-l, Interferon-gamma and TNF-alpha to the LAK supernatants prior to using them to activate monocytes. The above procedurewas then carried out as outlined with in each case control wells being set up with neutralising antibodies, monocytes and tumourtarget cells only to determine whether the antibodies themselves could alter tumour cell killing or TNF-alpha secretion. From the normal monocyte cytotoxicity graph, (**Figure 7a**), the monocytes when stimulated with IL-2 produced LAK supernatants showed increased cytotoxicity on 200Unit supernatants over those unstimulated supernatants of the corresponding day with 1000 and 2000 Units IL-2 doses also showing increased cytotoxicity. When the TNF-alpha secretion was examined it could again be seen that incubation of the monocytes with the 200, 1000, and

2000 Unit stimulated LAK supernatants produced a marked increase in TNF-alpha production.

The anti-CD3 stimulated LAK supernatants did not on the whole produce an equivalent amount of % generated cytotoxicity and TNF-alpha secretion was lower although, where it did occur, the 10 and 100 ng/ml dose anti-CD3 LAK supernatants did produce heightened TNF-alpha secretion to those unstimulated LAK supernatants of equivalent days.

When neutralising antisera for various cytokines was added to the supernatants prior to their incubation with the monocytes, they all brought about a decrease in both the % cytotoxicity generated and the amount of TNF-alpha secreted. The IL-1 and IFN-gamma antisera had the greatest effects as seen by the reduced bars on the graphs when compared with the whole, unmodified, supernatants, compare **Figures 7b, 7c and 7d**.

Graph showing the correlation between TNF-alpha secreted by the activated monocytes and % cytotoxicity generated

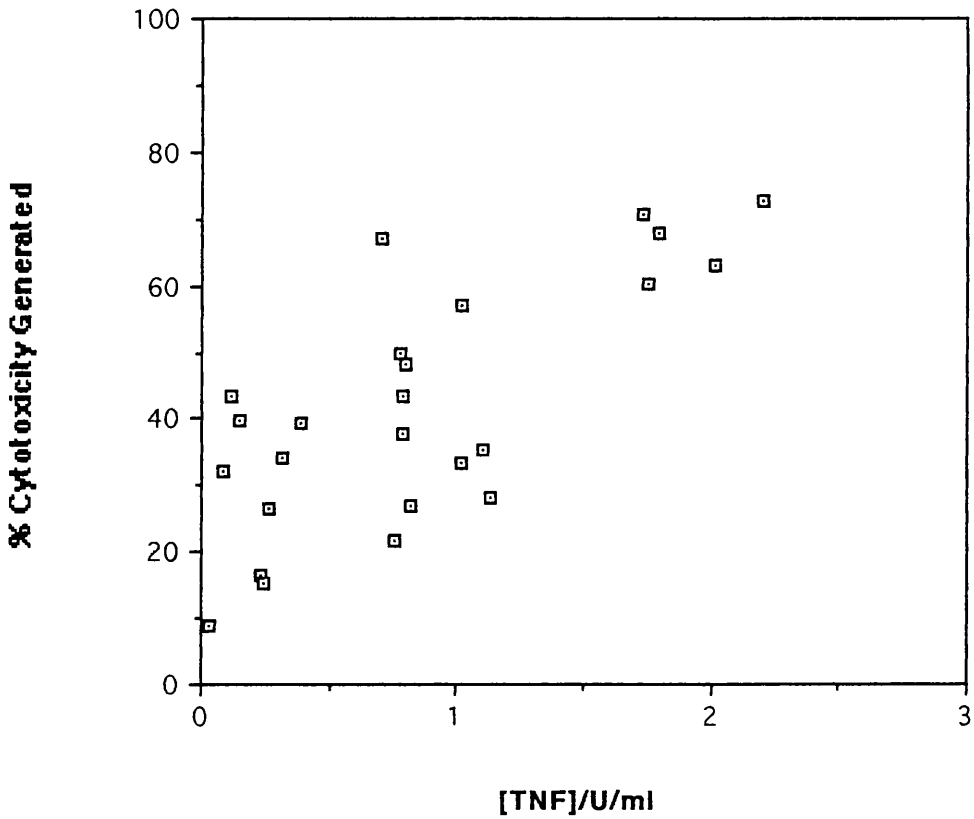
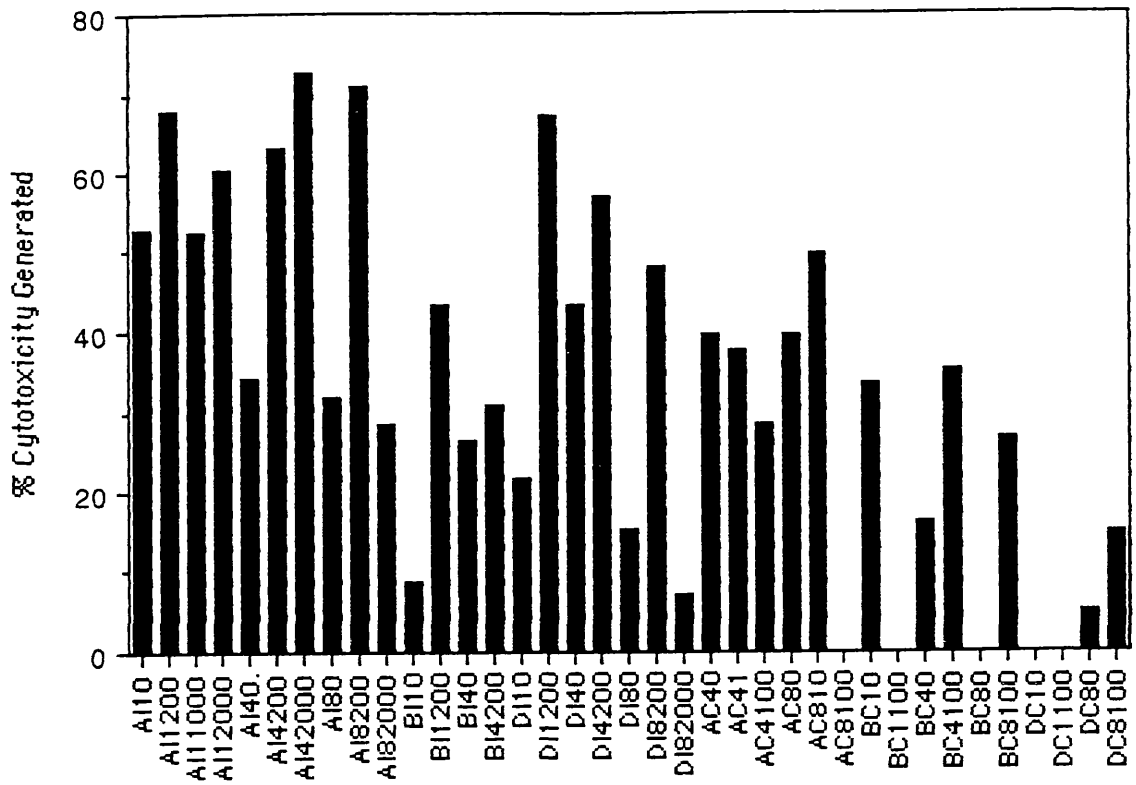


Figure 7a

% CYTOTOXICITY GENERATED AGAINST TUMOUR CELL TARGETS BY HUMAN PERIPHERAL BLOOD MONOCYTES STIMULATED WITH VARIOUS LAK CELL SUPERNATANTS



TNF-ALPHA PRODUCTION OF HUMAN PERIPHERAL BLOOD MONOCYTES WHEN STIMULATED WITH VARIOUS LAK CELL SUPERNATANTS

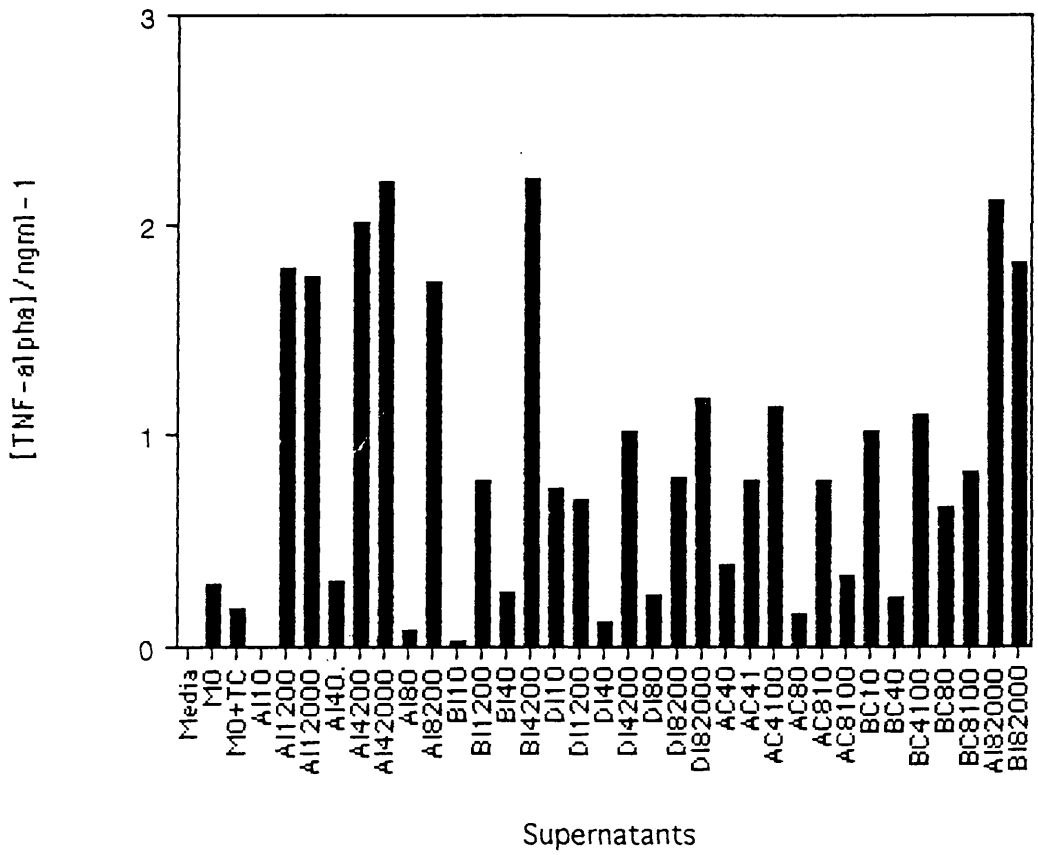
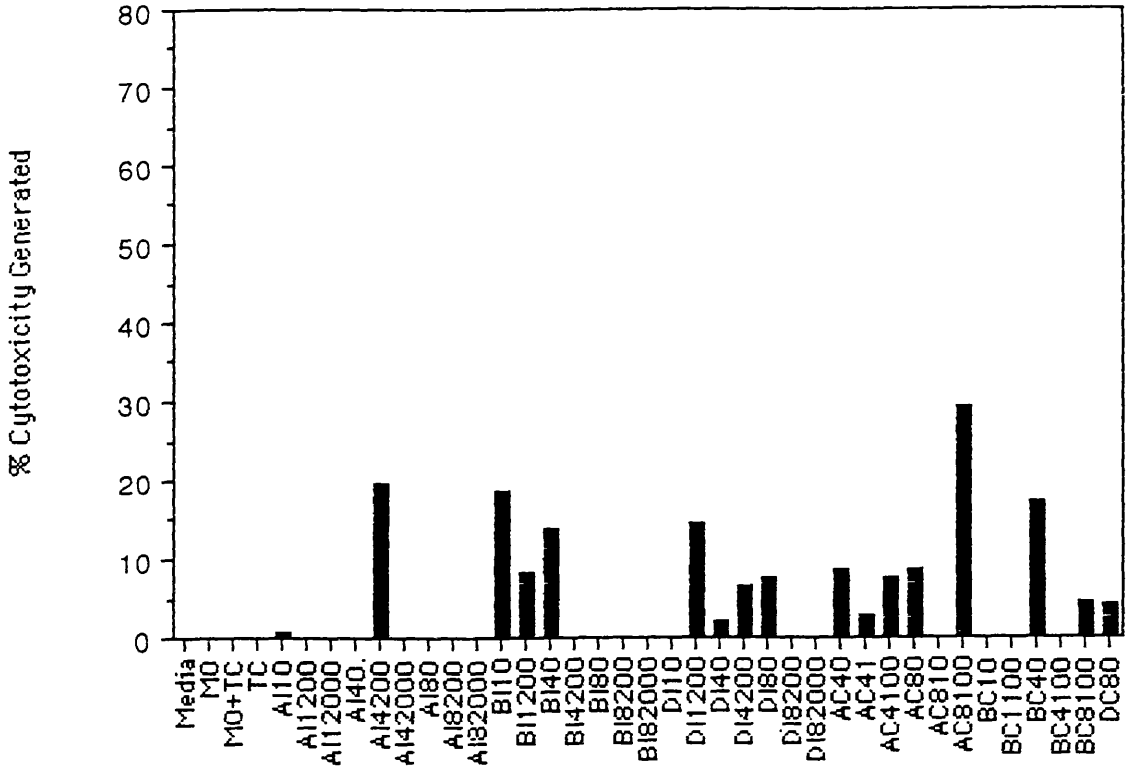


Figure 7b

% CYTOTOXICITY GENERATED AGAINST TUMOUR CELL TARGETS BY HUMAN PERIPHERAL BLOOD MONOCYTES STIMULATED WITH VARIOUS LAK CELL SUPERNATANTS (IFN-GAMMA DEPLETED)



TNF-ALPHA PRODUCTION OF HUMAN PERIPHERAL BLOOD MONOCYTES WHEN STIMULATED WITH VARIOUS LAK CELL SUPERNATANTS (IFN-GAMMA DEPLETED)

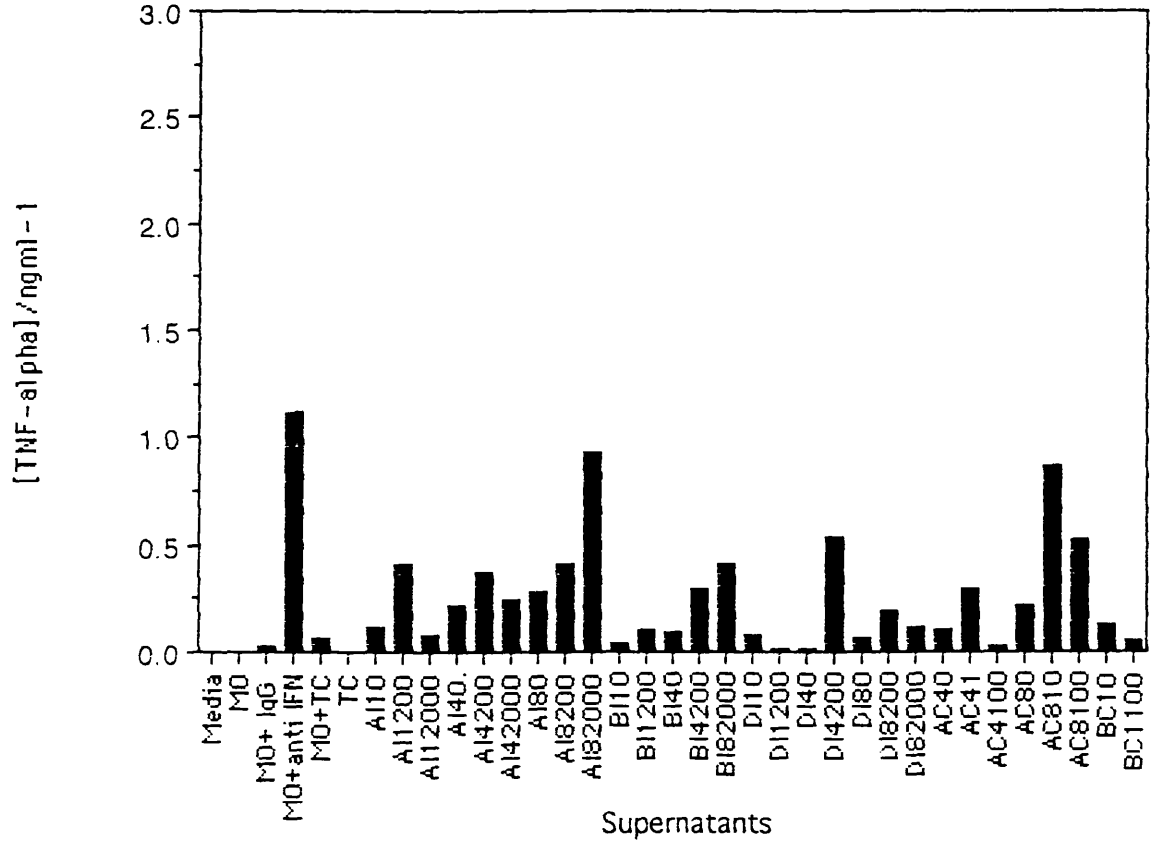
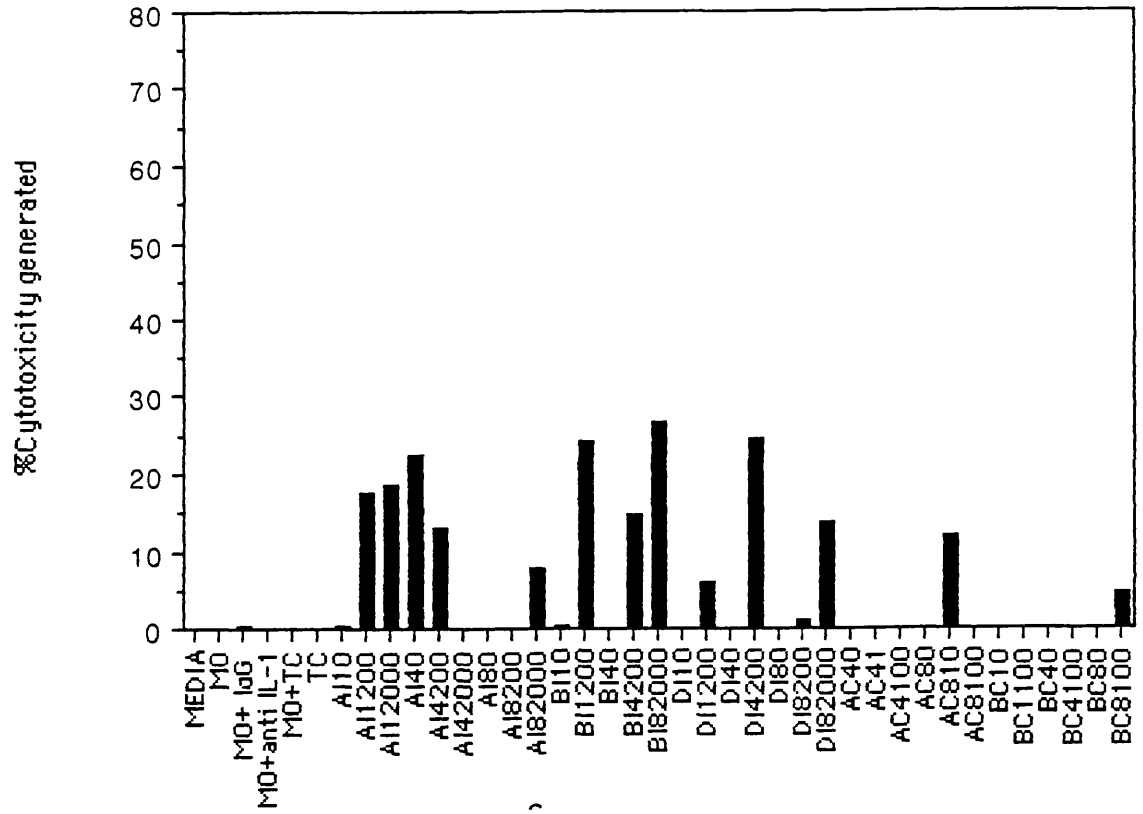


Figure 7c

% CYTOTOXICITY GENERATED AGAINST TUMOUR CELL TARGETS BY HUMAN PERIPHERAL BLOOD MONOCYTES STIMULATED WITH VARIOUS LAK CELL SUPERNATANTS (IL-1 DEPLETED)



TNF-ALPHA PRODUCTION OF HUMAN PERIPHERAL BLOOD MONOCYTES WHEN STIMULATED WITH VARIOUS LAK CELL SUPERNATANTS (IL-1 DEPLETED)

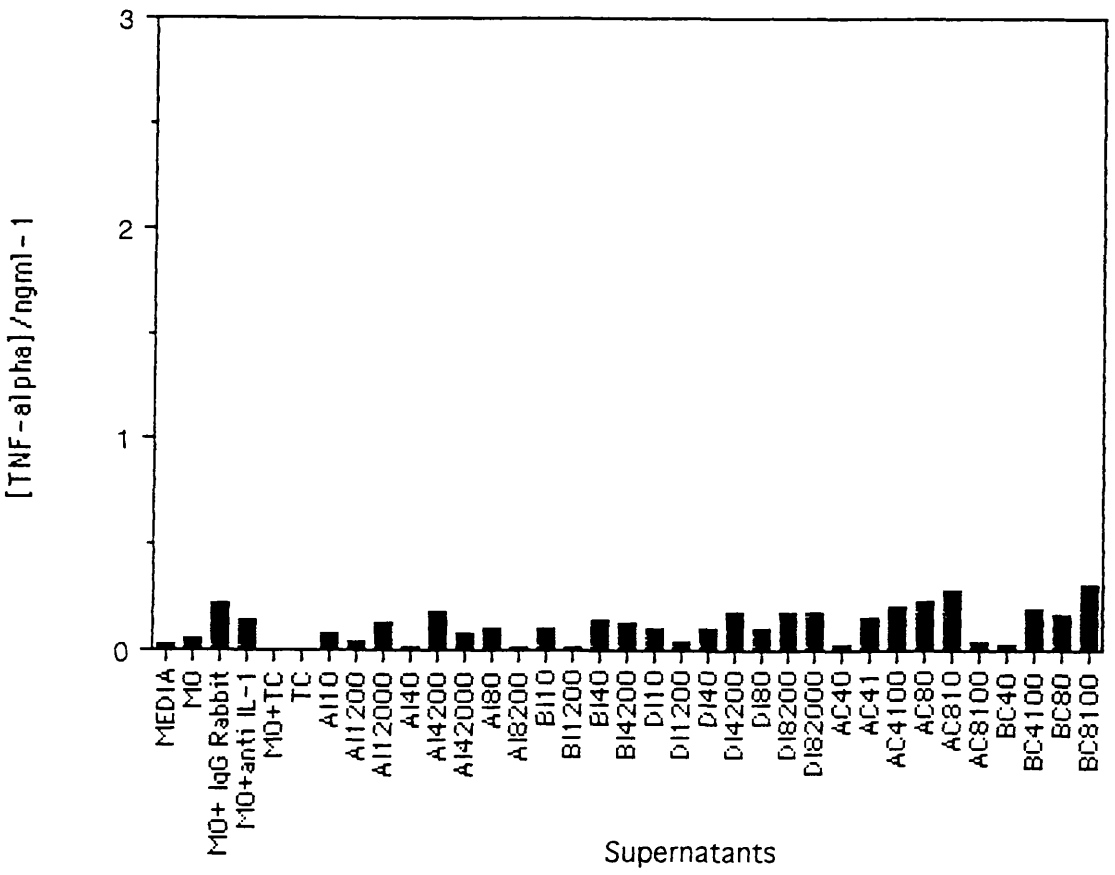
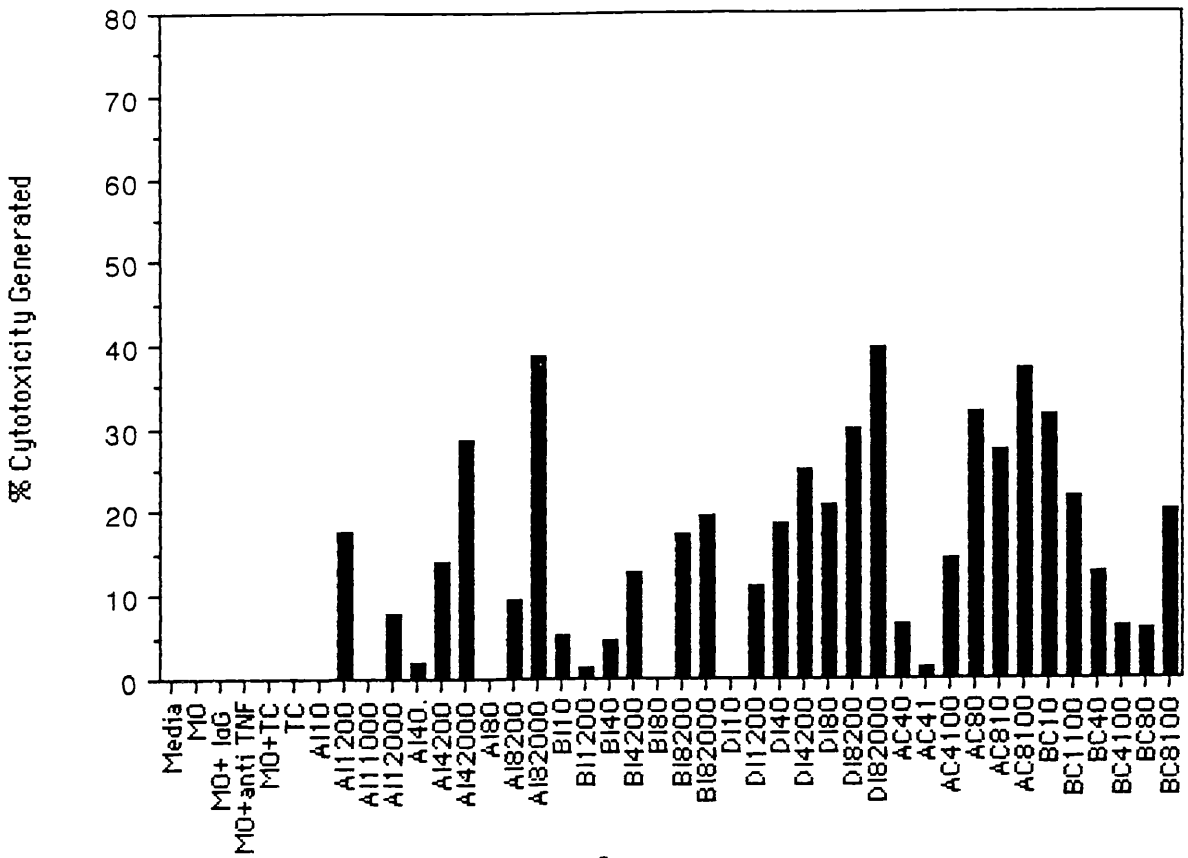
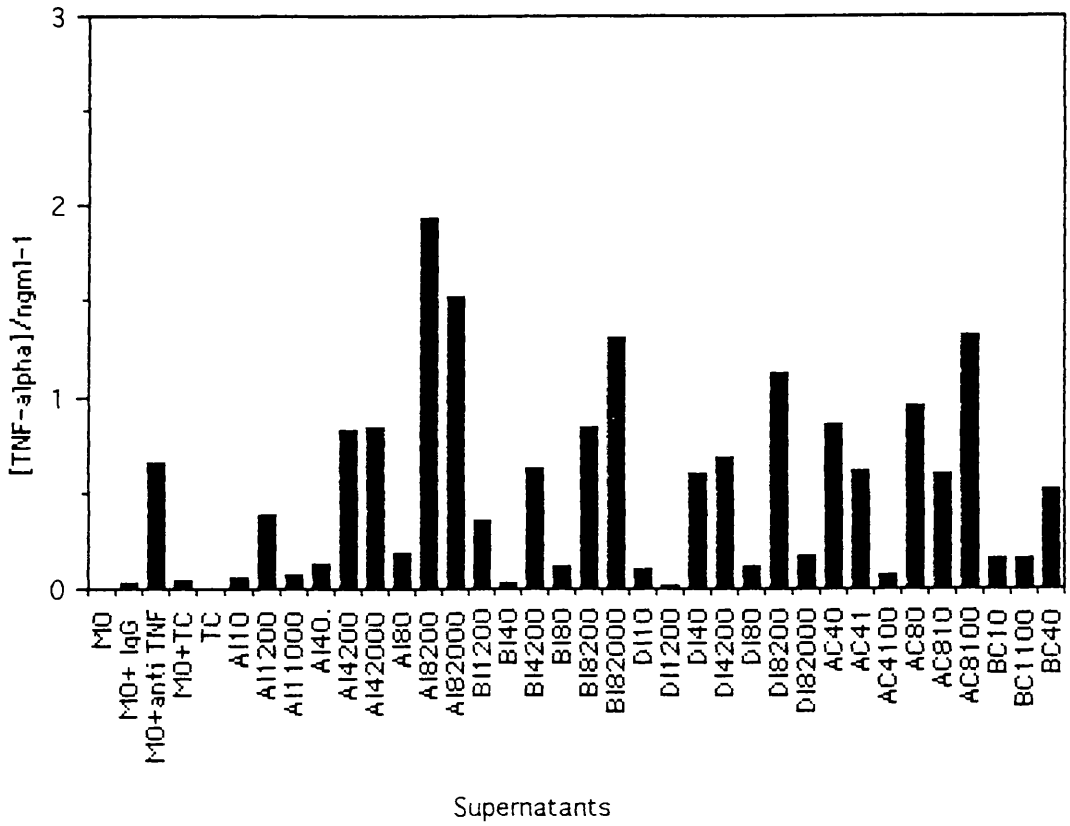


Figure 7d

% CYTOTOXICITY GENERATED AGAINST TUMOUR CELL TARGETS BY HUMAN PERIPHERAL BLOOD MONOCYTES STIMULATED WITH VARIOUS LAK CELL SUPERNATANTS (TNF-ALPHA DEPLETED)



TNF-ALPHA PRODUCTION OF HUMAN PERIPHERAL BLOOD MONOCYTES WHEN STIMULATED WITH VARIOUS LAK CELL SUPERNATANTS (TNF-ALPHA DEPLETED)



CHAPTER 5

DISCUSSION

5.1 General Discussion

5.2 Recent Literature Review and Future Work

5.1 : Discussion

It has been reported in several studies that peripheral blood monocytes cultured in direct contact with tumour cells are capable of causing tumour cytostasis and cytolysis (38,45). The addition of various exogenous cytokines such as interferon-gamma(30) and IL-1(38) have demonstrated an enhanced effect.

In the present study endogenous cytokine was to be produced by pre-stimulation of PBMC with IL-2 and anti-CD3 antibody. Thus only one dose would need to be used to give a the desired effect of monocyte tumour cell cytotoxicity. Of the many LAK supernatants which were originally produced, the 200U/ml of stimulating IL-2 for 4 days produced, the cocktail of cytokines which most ably induced monocyte tumour cytotoxicity in three of the four donors (A,B and D).

It was found that the U937Cell line behaved in a similar way to the donor monocytes in its ability to be stimulated by the LAK supernatants to become cytotoxic. Thus the effect seen was not just a one of freak occurrence but could be reproduced. However, small variations in cytotoxicity and cytokine secretion did exist between individual donors of both PBMC and monocytes, but as can be seen from the data the general trends remained the same.

On the whole, supernatants produced by stimulating PBMC with the anti-CD3 mAb did not stimulate monocytes to kill the tumour target cells. Any killing of the cells could have been due to carried over anti-CD3 present in the supernatants which would cause the monocytes to kill via ADCC. Donor C PBMC naturally secreted a high dose of TNF-alpha. This may have been responsible for a heightened general cytotoxicity but a lower specific cytotoxicity as that generated by stimulating cytokines, i.e. IFN-gamma and IL-l. It must also be noted that IFN-gamma and IL-l secretions are down-regulated by TNF-alpha in this donor. Wanidworanun et al. (73) reported that TNF-alpha causes monocytes to secrete IL-10 which in turn is able to downregulate TNF-alpha and interferon gamma secretion. Thus, levels of TNF-alpha produced initially in the LAK supernatants would have to be kept to a minimum to prevent this negative feedback mechanism. Therefore any monocyte cytotoxic stimulation which occurs due to the presence of these cytokines in donor C PBMC supernatants may be either totally or partially negated by the presence of TNF-alpha.

MHC class II antigen was upregulated by the LAK supernatants, predominantly those from the PBMC stimulated with 200U/ml of IL-2. IL-1

and interferon-gamma were at there highest concentrations in these supernatants which coincides with the findings of Ezekowitz et al. 1981 (74) who showed an increase in MHC class-II on monocyte cell membranes after stimulation with these cytokines. There was a significant correlation ($r=0.595$, $p=0.011$) between the expression of MHC class-II molecules on the surface of stimulated monocytes and the levels of IFN-gamma present in the activated supernatants. However there was no correlation between levels of MHC II expression on the monocytes and % generated tumour cytotoxicity The concentration of TNF-alpha the monocytes secreted was directly proportional to the level of generated cytotoxicity, therefore the most probable mechanism for tumour cell death. The % expression/intensity of the adhesion molecules LFA-1 and Mac-1 did not appear to change with the addition of the LAK supernatants.

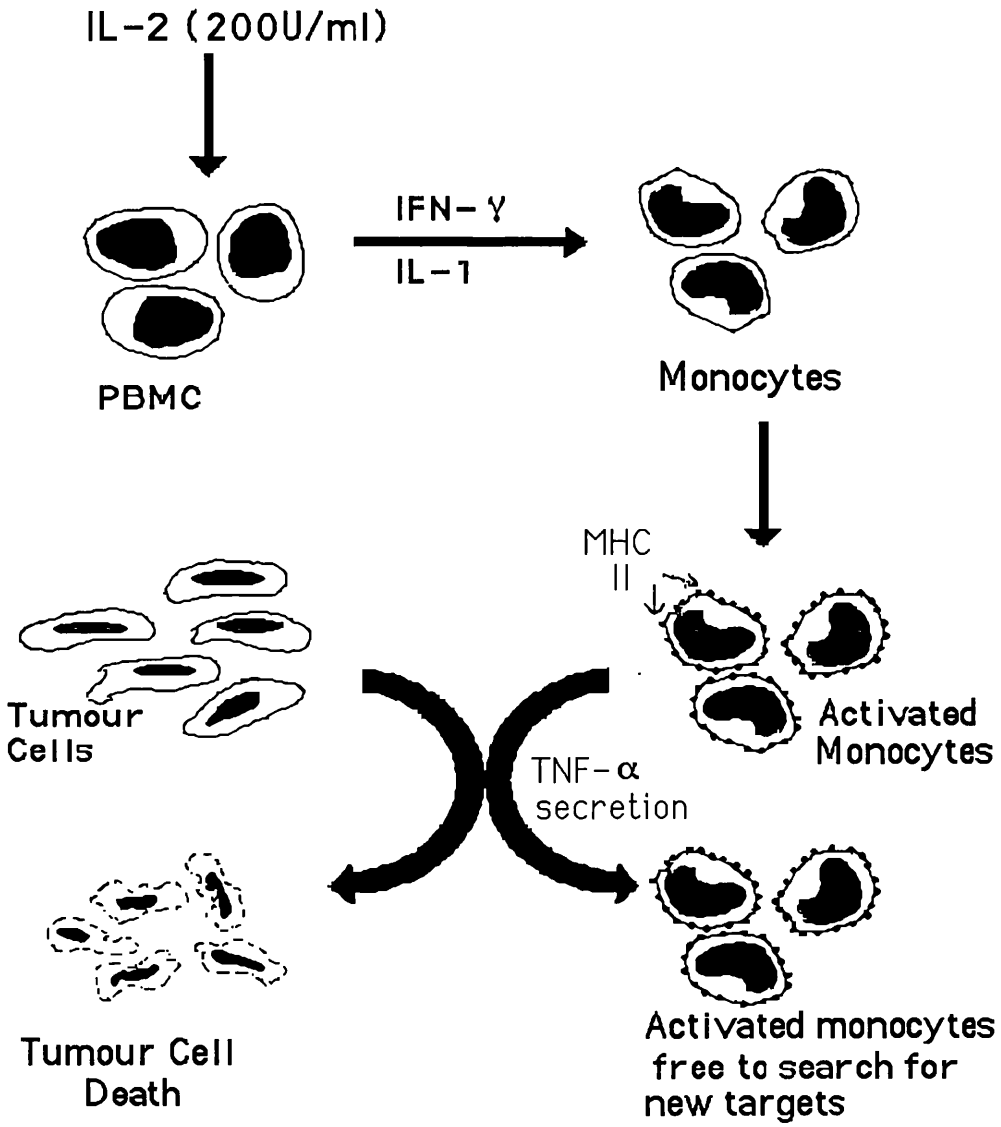
Therefore, in summary, the 200U/ml dose of IL-2 stimulated PBMC to secrete IFN-gamma and IL-1 in their highest concentrations. These cytokines then caused the upregulation of MHC II molecules on the surface of peripheral blood monocytes, thus activating them. The activated monocytes secreted a high concentration of TNF-alpha which correlated to the levels of generated monocyte mediated tumour cytotoxicity, thus a possible effector mechanism was observed (See Figure 8).

The positive results obtained here must be weighed up against negative side-effects of IL-2 therapy, which may have a dramatic effect in vivo. Firstly, the administration of IL-2 causes capillaries to leak (Ettinghausen et al. 1988 (75)); this can have a dramatic effect on the body as a whole. This occurrence has been postulated to enable phagocytes to enter the tissue easily from capillaries, so any administration of IL-2 must be at the tumour site thereby having a local effect while diluting systemic toxicity. TNF-alpha not only has a well documented anti-tumour response in animals but also has been shown to promote tumour cell adhesion to the peritoneum and so enhance the establishment of multiple tumours below the peritoneal surface in tumour-bearing mice (76). Finally Parry et al. 1992 (77) reported that monocytes secrete factors which cause the proliferation of tumour cells, therefore, in any supernatant added, these factors would have to be negated. The model proposed here would need to be examined in vivo to examine this trend and ascertain if the positive effects outweigh the negative side-effects. The main problem with working with the cytokine network and the immune system is the various cascades which are set into motion by the changing of one variable. Using the correct dose of IL-2 produces a cascade effect which ends in monocyte mediated cytotoxicity

invitro but as with most results observed when manipulating the immune system the knock on effects in vivo can produce a wide range of the is a whole new ball game.

FIGURE 8

A schematic representation of the events thought to be involved in the optimal stimulation by LAK supernatants, upon human monocyte tumour cell cytotoxicity



5.2 Recent Literature review and Future Work

Kuramitsu et al. discovered that TGF-beta could be used as a chemoattractant for LAK cells in vitro (79). If this could be used in humans, LAK cells could be targeted to the sight of tumours by either direct exogenous injection of the cytokine at the tumour site or by the use of antibodies attached to slow release liposomes containing the cytokine which have been engineered to target, tumour specific antigens. Heaton and Grimm used an antibody (HTac) which had specificity against the high/low affinity IL-2 receptor (IL-2R) of human PBMC. They found that when added to IL-2 activated PBMC there was a dramatic reduction in proliferation and secretion of the cytokines IFN-γ and TNF-α but no decrease in LAK activity. Another antibody (HMik) which was specific against the intermediate affinity IL-2 receptor (IL-2R) when used in a similar experiment, inhibited LAK activation and proliferation but had little effect on cytokine secretion (80). Therefore this data suggests LAK activity is mediated through the intermediate affinity IL-2R and cytokine secretion is mediated through the high low affinity complex, interaction between IL-2 and both of these complexes is required for the induction and maintenance of proliferative effects by human PBMC's.

Kirsch et al. 1994 demonstrated that IFN-gamma and TNF-alpha when used together activated human monocytes to kill tumour cells in a heightened way to either of the cytokines alone (81) . To examine If LAK activity could be heightened above that seen with IL-2 alone, IL-6, IL-7, IFN-alpha, and IFN-gamma were used in conjunction with optimal and sub-optimal doses of IL-2. Exposure with IL-6 and IL-7 with sub-optimal doses of IL-2 led to increased proliferation after stimulation this increase was not observed in cytotoxicity. Thus these cytokines could be examined in my experiments to produce heightened cytokine concentrations in the supernatants (82) . Fujiwara and Grimm in 1992 reported that a combination of IL-1 and IL-2 increased TNF-alpha synthesis in PBMC than stimulation with IL-2 alone(83). Tumour cell lines have been transduced using retroviruses in vitro TNF-alpha and IFN-gamma (84). If this transduction could be brought occur in vivo the tumour itself could stimulate the immune system to destroy itself.

One of the most commonly abnormalities able to be detected in human cancer is mutation of the p53 tumour suppresser gene. The function of the p53 gene is the induction of apoptotic cell death. Loss of p53 causes an increased resistance to radio- and chemo-therapeutic agents (85).

Moreover mutation of the tumour suppresser genes is thought to contribute to tumour cell growth by activating proteins which normally limit cell proliferation. (86). A recently cloned TNF family member (TNF-related apoptosis -inducing ligand (TRAIL))has been identified that induces apoptosis in a wide variety of cell lines of diverse origin. (87). This cytokine could be examined in my experiments to see if this was the mechanism through which the monocytes were killing the tumour cells.

To verify the effect observed from the results pure cytokines could be added to stimulate monocytes to reproduce the levels of cytotoxicity seen with that produced with the LAK supernatants. Cytokine synergy could also be examined by using antibody blocking against the relevant cytokines of the supernatants.

The anti-CD3 supernatants did not increase monocyte tumour cytotoxicity very much but contained IL-I. Thus this may be circumstantial evidence that other stimulatory factors are present in the IL-2 produced LAK supernatants. Such proteins could be other cytokines e.g. GM-CSF or IL-6, these could be examined in a similar manner to TNF-alpha, IFN-gamma and IL-I to quantify any role in which they may contribute to monocyte tumour cytotoxicity (stimulated by LAK supernatants).

Also the use of LAK supernatants could be evaluated in vivo by using a SCID or nude mouse model. Here the mice could be reconstituted with human PBMC and/or monocytes. IL-2 at the correct concentrations could be administered and the effect of LAK secretions upon monocyte mediated tumour cell killing could be examined. From the work carried out by Kluppen et al. 1992 (88) the site of administration would be local to the tumour. The extent of particular LAK cell secretions could be examined not only upon monocytes but also PBMC and monocytes thus the extent of monocyte and T cell cytotoxicity could simultaneously be examined.

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